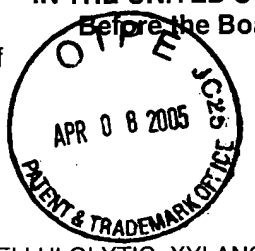
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Before the Board of Patent Appeals and Interferences

AF 18



In re Patent Application of: Debyser et al
Serial No. 09/403,625
Filed: February 7, 2000
Title: INHIBITORS OF CELLULOLYTIC, XYLANOLYTIC AND BETA-GLUCANOLYTIC ENZYMES

Atty Dkt. 550-392
C# M#
TC/A.U.: 1652
Examiner: Fronda
Date: April 8, 2005

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

☐ **Correspondence Address Indication Form Attached.**

☐ **NOTICE OF APPEAL**
Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the Examiner twice/finally rejecting applicant's claim(s). $500.00 (1401)/$250.00 (2401) $

☒ An appeal **BRIEF** is attached in the pending appeal of the above-identified application $500.00 (1402)/$250.00 (2402) $ 500.00

☐ Credit for fees paid in prior appeal without decision on merits -$ ( )

☐ A reply brief is attached. **(no fee)**

☐ Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s)
One Month Extension $120.00 (1251)/$60.00 (2251)
Two Month Extensions $450.00 (1252)/$225.00 (2252)
Three Month Extensions $1020.00 (1253/$510.00 (2253)
Four Month Extensions $1590.00 (1254/$795.00 (2254) $

☐ "Small entity" statement attached.

Less month extension previously paid on -$( )

TOTAL FEE ENCLOSED $ 500.00

Any future submission requiring an extension of time is hereby stated to include a petition for such time extension. The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our **Account No. 14-1140.** A duplicate copy of this sheet is attached.

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NIXON & VANDERHYE P.C.
By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: _____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Patent Application of

Debyser et al — Atty. Ref.: 550-392

Serial No. 09/403,625 — TC/A.U.: 1652

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For: INHIBITORS OF CELLULOLYTIC, XYLANOLYTIC AND BETA-GLUCANOLYTIC ENZYMES

* *

April 8, 2005

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APPEAL BRIEF

Sir:

Applicant hereby appeals the final rejection of claims 48-50, 52-56 and 65-68, in the Office Action dated November 18, 2004, and submits the present Appeal Brief pursuant to 37 CFR § 41.37.

Table of Contents

(1) REAL PARTY IN INTEREST

The real party in interest is K. U. LEUVEN RESEARCH & DEVELOPMENT, Groot Begijnhof 59, B-300 Leuven, Belgium, by way of an Assignment from the applicants, recorded in the U.S. Patent and Trademark Office on June 15, 2000, at Reel 010899, Frames 0448-04450.

(2) RELATED APPEALS AND INTERFERENCES

The appellant, the appellant's legal representative, and the assignee are not aware of any related prior or pending appeals or interferences or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(3) STATUS OF THE CLAIMS

Claims 48-57 and 65-68 are pending.

Claims 48-50, 52-56 and 65-68 have been finally rejected.

Claims 51 and 57 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claim. See, page 4 of the Office Action dated November 17, 2003 (Paper No. 23) and page 3 of the Office Action dated December 18, 2004.

Originally-filed claims 1-33 were amended and claims 34-47 added in a Preliminary Amendment Including Sequence Listing filed November 1, 1999. Claim 6 was amended in a Response to Restriction Requirement and Preliminary Amendment filed September 28, 2001. Claims 6-13 were further amended in an Amendment filed May 6, 2002. Claims 1-47 were canceled and claims 48-57 added in an Amendment Under Rule 116, filed December 17, 2002, which was entered with the filing of the Request for Continued Prosecution (RCE) on February 21, 2003. Claims 48-57 were amended and claims 58-64 added by way of an Amendment dated June 26, 2003 and claims 48-57 further amended, claims 58-64 canceled and claims 65-68 added by way of a Supplemental Amendment filed August 29, 2003.

An RCE (Request for Continued Examination) was filed July 19, 2004 with amendments to the specification.

No amendments to the claims have been filed in response to the Office Action dated November 18, 2004.

Claims 48-50, 52-56 and 65-68 are the subject of the present appeal.

A copy of all the rejected claims 48-50, 52-56 and 65-68, i.e., the claims involved in the appeal, is attached as a Claims Appendix, pursuant to Rule 41.37(c)(1)(viii).

(4) STATUS OF THE AMENDMENTS

An Amendment has not been filed in response to the final Office Action dated November 18, 2004.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

The presently claimed invention provides isolated protein or glycoprotein inhibitors of xylanase. See, page 1, lines 11-23 and originally-filed claim 1.

The inhibitors of the presently claimed invention may be used, for example, in different areas of food, feed and/or beverage technologies, such as malting and brewing, the production of animal feedstuffs such as to increase their conversion, the production of baked and/or extruded cereal products such as straight dough, sponge and dough and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles, and the production of starch derived syrups, sorbitol, xylose and/or xylitol. See, page 1, line 24 through page 2, line 7 of the application. The inhibitors may further be used, for example, to reduce syruping of refrigerated breads which is thought to be the result of deleterious breakdown of arabinoxylan, leading to a decrease in the water holding capacity of the dough over time caused by endogenous xylanases in wheat flour.

Of the claims on appeal, claims 48, 49 and 65-68 are independent claims. Claims 50 and 52-56 are dependent claims.

The presently claimed invention provides protein or glycoprotein inhibitors of xylanase which are described in the claims by combinations of the following physical and/or chemical properties:

(1) the claimed product is a protein or glycoprotein (see, for example, page 5, lines 9-10 of the specification, originally-filed claim 1 and all of the pending claims);

(2) the claimed product is an inhibitor of xylanase (see, for example, page 5, lines 24-25 of the specification, originally-filed claim 2 and all the pending claims);

(3) the claimed product is water-soluble (see, for example, page 5, line 25 of the specification, originally-filed claim 7 and all the pending claims);

(4) the claimed product is alkaline (see, for example, page 5, line 25 of the specification, and all of the pending claims);

(5) the claimed product comprises an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1 (see, for example, page 5, line 31 through page 6, line 5 and page 6, lines 11-16 of the specification, originally-filed claims 8 and 9, and independent claims 48 and 49, and claims 50-57 dependent there from);

(6) the claimed product has a pI of greater than about 7.0 (see, for example, page 5, lines 26-27 of the specification, originally-filed claim 15, and all the pending claims);

(7) the claimed product, as exemplified by the wheat extract, has a molecular weight of about 40-43 kDa as measured by SDS-PAGE (see, for example, page 6, lines 11-13, originally-filed claims 14 and 15, and all the pending claims);

(8) the claimed product, as exemplified by the wheat extract, resolves as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol (see, for example, page 6, lines 17-28 and page 19, line 14 through page 20, line16 of the specification, originally-filed claim 14 and independent claims 49, 65, 66, 67 and 68, and dependent claims 51, 53, 55 and 57);

(9) the claimed product is obtainable from a cereal plant or a cereal plant fraction thereof (see, for example, page 5, lines 16-20, originally-filed claims 3-4, and independent claims 65-68 and dependent claims 52-57) and more specifically, from wheat (see, for example, page 5, lines 19-20 and pages 18-20 of the specification, originally-filed claim 4, independent claims 65, 66, and 67, and dependent claims 54, 55, 56 and 57), rye and barley (see, for example, page 5, lines 19-20 of the specification, originally-filed claim 4, independent claims 66, and 67, and dependent claims 54, 55, 56 and 57), and triticale, sorghum, oats, maize and rice (see, for example, page 5, lines 19-20 of the specification, originally-filed claim 4, independent claim 66, and dependent claims 54, 55, 56 and 57) and/or

(10) the claimed product resolves as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol and the two separate bands comprise an amino acid

sequence of SEQ ID NO:1 and SEQ ID NO:2 (see, for example, page 5, line 29 through page 6, line 10, and page 20, lines 2-16 of the specification, originally-filed claim 8, and dependent claim 51).

The details of the claimed invention may be summarized, for example, in the following Table 1.

Table 1

	Claim No. [1]													
Recitation of specification and claims noted above:	48	49	50	52	53	54	55	56	65	66	67	68	51	57
(1) protein or glycoprotein	X	X	X	X	X	X	X	X	X	X	X	X	X	X
(2) inhibitor of xylanase	X	X	X	X	X	X	X	X	X	X	X	X	X	X
(3) water-soluble	X	X	X	X	X	X	X	X	X	X	X	X	X	X
(4) alkaline	X	X	X	X	X	X	X	X	X	X	X	X	X	X
(5)(i) N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1	X	X	X	X	X	X	X	X					X	X
(5)(ii) comprises amino acid sequence of SEQ ID NO:1			X					X					X	X
(6) pI of greater than about 7.0	X	X	X	X	X	X	X	X	X	X	X	X	X	X
(7) Molecular weight of about 40-43 kDa as measured by SDS-PAGE	X	X	X	X	X	X	X	X	X	X	X	X	X	X
(8) resolves as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol w/m.w. of 30 & 10 kDa		X			X		X		X	X	X	X	X	X
(9)(i) cereal plant or a cereal plant fraction thereof				X	X	X	X	X		X	X	X		X
(9)(ii) wheat						X	X	X	X	X	X			X
(9)(iii) rye and barley						X	X	X		X	X			X
(9)(iv) triticale, sorghum, oats, maize and rice						X	X	X		X				X
(10) resolves as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol and the two separate bands comprise an amino acid sequence of SEQ ID NO:1 and SEQ ID NO:2													X	X

[1] Claims 48, 49 and 65-68 are independent claims; claims 50 and 52-56 are dependent claims; and claims 51 and 57 have not been rejected.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following ground of rejection is presented for review:

Whether the invention of claims 48-50, 52-56 and 65-68 is supported by an adequate written description, as required by 35 U.S.C. § 112, first paragraph.

(7) ARGUMENT

The invention of claims 48-50, 52-56 and 65-68 is adequately described by the present application. The Section 112, first paragraph "written description", rejection of claims 48-50, 52-56 and 65-68 should be reversed. Consideration of the following in this regard is requested.

The presently claimed invention provides isolated protein or glycoprotein inhibitors of xylanase. See, page 1, lines 11-23 and originally-filed claim 1.

The inhibitors of the presently claimed invention may be used, for example, in different areas of food, feed and/or beverage technologies, such as malting and brewing, the production of animal feedstuffs such as to increase their conversion, the production of baked and/or extruded cereal products such as straight dough, sponge and dough and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles, and the production of starch derived syrups, sorbitol, xylose and/or xylitol. See, page 1, line 24 through page 2, line 7 of the application. The inhibitors may further be used, for example, to reduce syruping of refrigerated breads which is thought to be the result of deleterious breakdown of arabinoxylan, leading to a decrease in the water holding capacity of the dough over time caused by endogenous xylanases in wheat flour.

As noted above, the presently claimed invention provides protein or glycoprotein inhibitors of xylanase which are described in the claims by various combinations of the following physical and/or chemical properties: (1) the claimed product is a protein or glycoprotein (see, for example, page 5, lines 9-10 of the

specification, originally-filed claim 1 and all of the pending claims); (2) the claimed product is an inhibitor of xylanase (see, for example, page 5, lines 24-25 of the specification, originally-filed claim 2 and all the pending claims); (3) the claimed product is water-soluble (see, for example, page 5, line 25 of the specification, originally-filed claim 7 and all the pending claims); (4) the claimed product is alkaline (see, for example, page 5, line 25 of the specification, and all of the pending claims),; (5) the claimed product comprises an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1 (see, for example, page 5, line 31 through page 6, line 5 and page 6, lines 11-16 of the specification, originally-filed claims 8 and 9, and independent claims 48 and 49, and claims 50-57 dependent there from); (6) the claimed product has a pI of greater than about 7.0 (see, for example, page 5, lines 26-27 of the specification, originally-filed claim 15, and all the pending claims); (7) the claimed product, as exemplified by the wheat extract, has a molecular weight of about 40-43 kDa as measured by SDS-PAGE (see, for example, page 6, lines 11-13, originally-filed claims 14 and 15, and all the pending claims); (8) the claimed product, as exemplified by the wheat extract, resolves as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol (see, for example, page 6, lines 17-28 and page 19, line 14 through page 20, line16 of the specification, originally-filed claim 14 and independent claims 49, 65, 66, 67 and 68, and dependent claims 51, 53, 55 and 57); (9) the claimed product is obtainable from a cereal plant or a cereal plant fraction thereof (see, for example, page 5, lines 16-20, originally-filed claims 3-4, and independent claims 65-68 and dependent claims 52-57) and more

specifically, from wheat (see, for example, page 5, lines 19-20 and pages 18-20 of the specification, originally-filed claim 4, independent claims 65, 66, and 67, and dependent claims 54, 55, 56 and 57), rye and barley (see, for example, page 5, lines 19-20 of the specification, originally-filed claim 4, independent claims 66, and 67, and dependent claims 54, 55, 56 and 57), and triticale, sorghum, oats, maize and rice (see, for example, page 5, lines 19-20 of the specification, originally-filed claim 4, independent claim 66, and dependent claims 54, 55, 56 and 57) and/or (10) the claimed product resolves as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol and the two separate bands comprise an amino acid sequence of SEQ ID NO:1 and SEQ ID NO:2 (see, for example, page 5, line 29 through page 6, line 10, and page 20, lines 2-16 of the specification, originally-filed claim 8, and dependent claim 51).

The details of the claimed invention may be summarized, for example, in the above Table 1.

As briefly detailed above, the present application describes the claimed xylanase inhibitors with reference to physical and chemical characteristics. Moreover, the xylanase inhibitors of the claimed invention are exemplified by a description in the Examples of the specification. The exemplified inhibitors include inhibitors from wheat, barley and rye (i.e., "WF" or wheat flour, "RF" or rye flour, and "BWM" or barley whole meal, extracts; see, page 21, lines 19-24, for example).

In further characterizing the wheat extract, the appellants described, as a species of their invention, a xylanase inhibitor which contains a specific N-terminal

amino acid sequence (i.e., a 14 amino acid sequence of SEQ ID NO:1[2] which was discovered in the wheat species of the invention). The appellants further describe their invention as xylanase inhibitors which include N-terminal amino acid sequences which, along with the other claimed identifying characteristics, are 70% homologous to SEQ ID NO:1 (see, page 6, lines 11-16, for example, of the specification).

Moreover, the appellants have described the wheat extract species as containing a further specific amino acid sequence (i.e., a 17 amino acid sequence of SEQ ID NO:2) which was accessible to sequencing after ß-mercaptoethanol reduction of the wheat species. See, page 20, lines 2-16, for example, of the specification.

The Examiner is understood to believe that the specification only describes, within the requirements of 35 USC § 112, first paragraph, xylanase inhibitors which include SEQ ID NOs: 1 and 2.

The Examiner's assertion that the specification allegedly only describes xylanase inhibitors containing SEQ ID NOs: 1 and 2 is inappropriate in that the Examiner's interpretation of the specification focuses only on the exemplified embodiment of the wheat xylanase inhibitor and, at a minimum, fails to acknowledge the separate exemplifications of the barley and rye inhibitors of the examples, as well as the broader description of the genus of inhibitors of the specification.

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[2] SEQ ID NO:1 contains 14 amino acids which includes as amino acid 12 an Xaa which is preferably Asp. See, page 20, lines 6-8, for example, of the specification.

Reversal of the Section 112, first paragraph "written description", rejection is requested and consideration of the following and attached are requested in this regard.

The present application exemplifies wheat (WF), barley (BWM) and rye (RF) xylanase inhibitors, as noted above. See, page 20, line 18 to page 21, line 24 of the specification. Independent claims 65-67 and dependent claims 54-56 provide wheat, barley and rye xylanase inhibitors, as described in the specification.

The application further includes the following description of the claimed inhibitors:

> "According to a preferred embodiment of the present invention, the inhibitor is a xylanase inhibitor which is typically water-soluble alkaline proteinaceous species, having a pI (i.e. -log of the isoelectric point) of greater than about 7.0. The xylanase inhibitor molecular weight as determined by SDS-page is typically 40-43 kDa." See, page 5, lines 23-28 of the specification.

Moreover, the inhibitors of the invention are separately described in the specification as follows:

> "The N-terminal sequence of the 40-43 kDa protein or glycoprotein has not been described until now and is typically as follows: SEQ ID No. 1: Lys-Gly-Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Xaa-Thr-Ala, wherein Xaa being preferably Asp. ... Therefore, the present invention is also related to an inhibitor with an SDS-page molecular weight of typically 40-43 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 1." See, page 5, line 31 through page 6, line 3 and page 6, lines 11-16 of the specification (emphasis added).

Accordingly, the specification describes xylanase inhibitors containing an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1 and exemplifies a wheat xylanase inhibitor which contains an N-terminal amino acid sequence of SEQ ID NO:1. The appellants submit therefore that the alternative recitations of property or characterizing factors (5)(i) or (9)(i) of the above Table 1 are adequate to sufficiently describe the N-terminal sequence of presently claimed and disclosed invention. These properties are recited individually or in combination in rejected claims 48-50, 52-56 and 66-68. As noted above, independent claim 65 provides a wheat xylanase inhibitor.

Thus, the present application not only describes proteinaceous xylanase inhibitors present in plants (and in particular cereals), but also teaches the isolation of such an inhibitor from wheat, barley and rye. The appellants were clearly in possession of wheat, barley and rye xylanase inhibitors at the time the application was filed. Moreover, the appellants clearly described that their discovery of wheat, barley and rye xylanase inhibitors were exemplifications of the claimed cereal xylanase inhibitors.

The appellants further described their exemplified wheat xylanase inhibitor by including the N-terminal amino acid sequence of this inhibitor, i.e., SEQ ID NO: 1. The appellants further described their exemplified wheat xylanase inhibitor by a partial sequence (i.e., SEQ ID NO:2) after reduction with β-mercaptoethanol.

The appellants further partial sequence description of their exemplified wheat xylanase inhibitor should not detract from their extensive description of the more broadly claimed protein or glycoprotein xylanase inhibitors which are alkaline, water soluble, have a pI of greater than about 7.0, have a molecular weight of 40-43 kDa as measured by SDS PAGE and possess at least one of the following properties: contains an amino acid sequence of SEQ ID NO:1, contains an N-terminal sequence which is at least 70% homologous to SEQ ID NO:1 and resolves as two separate bands on SDS PAGE after reduction with β-merceptoethanol (see claim 68).

As a further example of claimed inhibitors obtainable from barley, the appellants note that such an inhibitor is specifically disclosed in WO 01/98474 (copy previously submitted with the appellants' Amendment of June 26, 2003 and listed on the PTO 1449 Form filed June 26, 2003, return of an initialed copy of which, pursuant to MPEP § 609, is again requested). The U.S. national phase of WO 01/98474 has been assigned U.S. Serial No. 10/311,886.

The barley inhibitor (designated HvXI in the attached WO 01/98474 document) specifically described in WO 01/98474 comprises an N-terminal amino acid which is 78.6% homologous[3] with that of SEQ ID NO:1 of the present application. The barley

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[3] Homology calculated using:
http://searchlauncher.bcm.tmc.edu/seq-search/alignment.html
Results of SIM with:
Sequence 1: HvXI 30, KALPVLAPVTKDAATSLYTI (20 residues)
Sequence 2: TAXI 30N, KGLPVLAPVTKXTA (14 residues (SEQ ID NO:1 of present application))
using the parameters:
Comparison matrix: BLOSUM62
Number of alignments computed: 20
Gap open penalty: 12

xylanase inhibitor exemplified in WO 01/98474 therefore is a species which exemplifies the invention of the appealed claims.

The appellants have also determined that the isolated barley and rye xylanase inhibitors do not contain SEQ ID NO:2 of the present application.

The identification of the barley xylanase inhibitor in the present application and subsequent elucidation of the sequence therefor serves as evidence that a further species of the presently claimed genus or subgenus exists, as described by the appellants in the present specification.

Moreover, with specific regard to the claimed recitation of the claimed inhibitor having an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1, the barley sequence serves as evidence that a xylanase inhibitor, as claimed, having an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1, was described by the present specification and has been further specifically identified by a skilled person following the teachings of the present specification.

The recited N-terminal sequence having a percent homology of at least 70% to SEQ ID NO:1 is supported by an adequate written description.

One of ordinary skill will appreciate that SEQ ID NO:1 of the present application is 14 amino acids in length. It is well-known in the present art to describe

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Gap extension penalty: 4
78.6% identity in 14 residues overlap; Score: 52.0; Gap frequency: 0.0%
HvXI 30, 1 KALPVLAPVTKDAA
TAXI 30N, 1 KGLPVLAPVTKXTA

protein sequences by a relative percent homology or identity to a base sequence. Such descriptions, and the desire for a simple means to make such calculations, led to the development and ready availability of, for example, the BESTFIT program.[4]

The U.S. Patent Office has granted at least 7 patents wherein the term "percent homology" is recited in a claim in the biotechnology area. Moreover, the Patent Office has granted at least 20 patents wherein the term BESTFIT is recited as an example of calculation of the identity or homology in the biotechnology area.[5]

The present specification provides a functional and a structural description of the claimed inhibitors. The structural similarity of the proteins of the present claims, which reference a percent homology and a base comparison sequence, will be appreciated by one of ordinary skill in the art and has been recognized by the Patent Office (see, previously submitted and above-noted appendices) as providing an adequate written description of amino acid sequences.

The Patent Office's "current understanding... regarding the written description requirement of 35 U.S.C. 112, ¶1" (see, 66 FR 1099, Friday, January 5, 2001 (copy

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[4] A list of the first 500 of 892 U.S. Patents issued from 1976 to a recent search date which include the words BESTFIT and protein were previously submitted as Appendix A to the Amendment filed August 29, 2003 as an indication of the prevalence of the terms in the field of biotechnology. Copy attached as Evidence Appendix (b). Each of the listed patents has not been reviewed in detail to assure the context in the listed patent is the same as used in the claims which are the subject of the present appeal. See, Appendix B attached to the Amendment filed August 29, 2003 for a description of the BESTFIT program. Copy attached as Evidence Appendix (c).

[5] Appendix C attached to the Amendment filed August 29, 2003 provides a list of the noted 27 patents, representative claims of each patent with the noted recitation and, in italics, the description of the relevant portions of the specification of each listed patent which describes the use of percent homology or the BESTFIT program as being routine. Copy attached as Evidence Appendix (d). Many of these patents were granted to Human Genome Sciences and contain the same or very similar, limited "boiler plate" text which is repeated in multiple applications.

attached as Appendix D to the Amendment filed August 29, 2003 and attached hereto as Evidence Appendix (e)) states that

> "An applicant may show possession of an invention by disclosure of drawings[39] or structural chemical formulas[40] that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole. The description need only describe in detail that which is new or not conventional.[41] This is equally true whether the claimed invention is directed to a product or a process.
>
> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics[42] which provide evidence that applicant was in possession of the claimed invention,[43] i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.[44] What is conventional or well-known to one of ordinary skill in the art need not be disclosed in detail.[45] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.[46]" Id. at 1106.

The indicated footnotes 39-46 further support this "understanding" of the Patent Office based on Federal Circuit, CCPA and other case law as follows:

> [39]*See, e.g., Vas-Cath*, 935 F.2d at 1565, 19 USPQ2d at 1118 ("drawings alone may provide a 'written description' of an invention as required by § 112"); *In re Wolfensperger*, 302 F.2d 950, 133 USPQ 537 (CCPA 1962) (the drawings of applicant's specification provided sufficient written descriptive support for the claim limitation at issue); *Autogiro Co. of America v. United States*, 384 F.2d 391, 398, 155 USPQ 697, 703 (Ct. Cl. 1967) ("In those instances where a visual representation can flesh out

words, drawings may be used in the same manner and with the same limitations as the specification.").

[40]*See e.g., Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus.").

[41]*See Hybritech v. Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp. v. General Electric Co.*, 107 F.3d at 1549, 41 USP!2d at 1805 (source code description not required).

[42]For example, the presence of a restriction enzyme map of a gene may be relevant to a statement that the gene has been isolated. One skilled in the art may be able to determine when the gene disclosed is the same as or different from a gene isolated by another by comparing the restriction enzyme map. In contrast, evidence that the gene could be digested with a nuclease would not normally represent a relevant characteristic since any gene would be digested with a nuclease. Similarly, isolation of an mRNA and its expression to produce the protein of interest is strong evidence of possession of an mRNA for the protein.

For some biomolecules, examples of identifying characteristics include a sequence, structure, binding affinity, binding specificity, molecular weight, and length. Although structural formulas provide a convenient method of demonstrating possession of specific molecules, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. For example, unique cleavage by particular enzymes, isoelectric points of fragments, detailed restriction enzyme maps, a comparison of enzymatic activities, or antibody cross-reactivity may be sufficient to show possession of the claimed invention to one of skill in the art. *See Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966 ("written description" requirement may be satisfied by using" such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention").

[43]A definition by function alone "does not suffice" to sufficiently describe a coding sequence "because it is only an indication of what the gene does, rather than what it is." *Eli Lilly*, 119 F.3 at 1568, 43 USPQ2d at 1406. *See also Fiers*, 984 F.2d

at 1169-71, 25 USPQ2d at 1605-06 (discussing *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991)).

[44]If a claim limitation invokes 35 U.S.C. 112, ¶ 6, it must be interpreted to cover the corresponding structure, materials, or acts
in the specification and "equivalents thereof." See 35 U.S.C. 112, ¶ 6. See also *B. Braun Medical, Inc. v. Abbott Lab.*, 124 F.3d 1419, 1424, 43 USPQ2d 1896, 1899 (Fed. Cir. 1997). In considering whether there is 35 U.S.C. 112, ¶ 1, support for a means- (or step) plus-function claim limitation, the examiner must consider not only the original disclosure contained in the summary and detailed description of the invention portions of the specification, but also the original claims, abstract, and drawings. A means- (or step-) plus-function claim limitation is adequately described under 35 U.S.C. 112, ¶ 1, if: (1) The written description adequately links or associates adequately described particular structure, material, or acts to the function recited in a means- (or step-) plus-function claim limitation; or (2) it is clear based on the facts of the application that one skilled in the art would have known what structure, material, or acts perform the function recited in a means- (or step-) plus- function limitation. Note also: A rejection under 35 U.S.C. 112, ¶ 2, "cannot stand where there is adequate description in the specification to satisfy 35 U.S.C. 112, first paragraph, regarding means-plus-function recitations that are not, per se, challenged for being unclear." *In re Noll*, 545 F.2d 141, 149, 191 USPQ 721, 727 (CCPA 1976). See *Supplemental Examination Guidelines for Determining the Applicability* of 35 U.S.C. 112, ¶ 6, 65 FR 38510, June 21, 2000.

[45]See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94.

[46]*See, e.g., Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (starting "the description need not be in *ipsis verbis* (i.e., "in the same words"] to be sufficient")." Id. at 1109-1110. (Emphasis added.)

As noted above in footnote 40, the Patent Office confirmed that the court in *Eli Lilly* found that claims involving generic formula usually indicate with specificity what the generic claims encompass. The court confirmed that one of ordinary skill in the

art can usually distinguish such a formula from others and can identify many of the species that the claims encompass. Given these facts, the *Eli Lilly* court concluded that "such a formula is normally an adequate written description." The Patent Office reliance on *Lockwood* above is also of particular relevance. A copy of the *Eli Lilly* decision is attached hereto as Evidence Appendix (f) for the convenience of the Board.

In the present application, the appellants have described, and recited in the claims, a protein or glycoprotein, which is an inhibitor of xylanase, which is water soluble, which is an alkaline protein or glycoprotein, which has a pI of greater than about 7.0, and which has a molecular weight of about 40-43 kDa as measured by SDS-PAGE. The claimed protein or glycoprotein is further described in the claims by at least (1) reference to an N-terminal amino acid sequence and a percent identity (see, independent claims 48 and 49 and dependent claims 50 and 52-56), or (2) a requirement that the inhibitor be a wheat xylanase inhibitor (see, independent claims 66 and 67) or (3) a requirement that the xylanase inhibitor resolves as two separate bands on SDS PAGE after reduction with β-mercaptoethanol with molecular weights of about 30 kDa and about 10 kDa (see, indepdnent claim 68), which allow one of ordinary skill to distinguish the generic formula of the claims from other protein sequences. One of ordinary skill can identify many species that the claims encompass. Given the conclusions of the *Eli Lilly* court, the appellants respectfully submit that the generic formulas of the claims and the specification provide an adequate written description of the claimed invention.

The issue before the *Eli Lilly* court, which was not mentioned in the footnote of the Patent Office's "written description" analysis, was whether even more generic statements, "such as 'vertebrate insulin cDNA' or 'mammalian insulin cDNA', without more," is an adequate' written description. See, 43 USPQ2d 1406 (emphasis added).

The *Eli Lilly* court found that such a generic recitation was not an adequate written description.

> "because it does not distinguish the claimed genus from others except by function. It does not specifically define any of the genes that fall within its definition. It does not specifically define any of the genes that fall within its definition. It does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus. A definition by function, as we have previously indicated, does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is. *See Fiers*, 984 F.2d at 1169-71, 25 USPQ2d at 1605-06 (discussing *Amgen*)." Id. (Emphasis added.)

As noted above, the claims of the present application recite a number of structural and functional features, as well as, in many of the claims, a reference sequence and a percent identity, as well as a molecular weight and other distinguishing features which allow one of ordinary skill to distinguish the protein of the claimed invention from other proteins.

The appellants respectfully submit that the present specification demonstrates possession of the claimed invention by, for example, disclosure of reference sequence (i.e., SEQ ID NO: 1) and the distinguishing features coupled with the

functional characteristics recited in the claims. An ordinarily skilled artisan would have understood that the appellants were in possession of the claimed invention at the time of filing.

Beyond the Patent Office "understanding" of the requirements of the Section 112, first paragraph, written description, requirement, as detailed above, the Patent Office has issued Training Materials

> "designed to aid PTO's patent examiners in applying the interim written description... guidelines in a uniform and consistent manner to promote the issuance of high quality patents. The training materials will also assist patent applicants in responding to the PTO when... written description issues are raised during the examination of a patent application." See, Press Release #00-15, USPTO, March 1, 2000 (www.uspto.gov/web/offices/com/speeches/00-15.html) (copy attached as Appendix E to the Amendment filed August 29, 2003, and attached hereto as Evidence Appendix (e)).

The Written Description Training Materials (http://www.uspto.gov/web/offices/pac/writtendesc.pdf) offer the following Example 14 "Product by Function":

> **"Example 14: Product by Function**
> **Specification:** The specification exemplifies a protein isolated from liver that catalyzes the reaction of **A→ B.** The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO: 3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.
> **Claim:**

A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A →B.

Analysis:

A review of the full content of the specification indicates that a protein having SEQ ID NO: 3 or variants having 95% identity to SEQ ID NO: 3 and having catalytic activity are essential to the operation of the claimed invention. The procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity. Moreover, procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art. A review of the claim indicates that variants of SEQ ID NO: 3 include but are not limited to those variants of SEQ ID NO: 3 with substitutions, deletions, insertions and additions; but all variants must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO: 3.

Additionally, the claim is drawn to a protein which **comprises** SEQ ID NO: 3 or a variant thereof that has 95% identity to SEQ ID NO: 3. In other words, the protein claimed may be larger than SEQ ID NO: 3 or its variant with 95% identity to SEQ ID NO: 3. It should be noted that "having" is open language, equivalent to "comprising". The claim has two different generic embodiments, the first being a protein which comprises SEQ ID NO: 3 and the second being variants of SEQ ID NO: 3. There is a single species disclosed, that species being SEQ ID NO: 3.

A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious. There is actual reduction to practice of the single disclosed species.

The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.

> **Conclusion:** The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.

The claims at issue in the present application relate to a protein or glycoprotein inhibitor which may be obtainable from a cereal plant or fraction thereof, such as from wheat, rye, triticale, barley, sorghum, oats, maize and rice. The "catalytic activity" described in the above-quoted Example 14 may be seen as analogous to the functional recitations in the present claims, as noted above. Moreover, the present claims further describe the claimed inhibitor by reference to a characteristic molecular weight. The "SEQ ID NO: 3" of the above quoted Example 14, may be seen as analogous to the SEQ ID NO: 1 of the pending claims. Finally, the "variants of the protein" discussed in the above-quoted Example 14 may be analogous to the proteins of the presently claimed invention which contain an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1. As with the above-quoted Example 14, the appellants submit that methods of making and identifying and testing proteins of the claimed genus are well know and would not require undue experimentation from the teachings of the specification and the generally advanced level of skill in the art.

The Patent Office's analysis and "understanding" of the "written description" requirements of 35 U.S.C. § 112, first paragraph, and assistance to examiners and applicants in applying the law, as expressed through the Training Materials, all

support the appellants belief that the presently claimed invention is supported by an adequate written description.

(7)(a) ARGUMENT pursuant to 37 CFR § 41.67(vii), ¶2, 2nd sentence - rejection of claim 65 argued separately

Claim 65 should be considered separately with regard to the Section 112, first paragraph "written description", rejection as the specification exemplifies a wheat xylanase inhibitor such that the further recitation of SEQ ID NO:1 and SEQ ID NO:2, in addition to the eight (8) characterizing features of claim 65 should not be required. The appellants were in possession of a wheat species, as claimed and exemplified in the specification. Further arguments in this regard are provided above.

(7)(a) ARGUMENT pursuant to 37 CFR § 41.67(vii), ¶2, 2nd sentence - rejection of claims 66 and 67 argued separately

Claims 66 and 67 should be considered separately with regard to the Section 112, first paragraph "written description", rejection as the specification exemplifies a wheat xylanase, as an example of a cereal xylanase inhibitor, such that the further recitation of SEQ ID NO:1 and SEQ ID NO:2, in addition to the eight (8) characterizing features of claims 66 and 67, should not be required. The specification further demonstrates that embodiments of the claimed inhibitors are present in both barley (BWM) and rye (RF) (see page 20, line 18 to page 21, line 24, and in particular to line 1 of page 21, of the specification). The appellants were in possession of a wheat, barley and rye

species which are submitted to adequately describe the xylanase inhibitors of claims 66 and 67. Further arguments in this regard are provided above.

(7)(a) ARGUMENT pursuant to 37 CFR § 41.67(vii), ¶2, 2nd sentence-rejection of claim 68 argued separately

Claim 68 should be considered separately with regard to the Section 112, first paragraph "written description", rejection as the specification is submitted to adequately describe protein or glycoprotein, cereal xylanase inhibitors which are water soluble, alkaline, have a pI of greater than about 7.0, which have a molecular weight to about 40-43 kDa as measured by SDS PAGE and resolves as two separate bands on SDS PAGE after reduction with β-mercaptoethanol with molecular weights of about 30 kDa and about 10 kDa. These distinguishing characteristics are exemplified by the species of the application and are submitted to be sufficient to at least establish the above-quoted requirements of written description articulated by the *Eli Lilly* court. Further arguments in this regard are provided above.

Reversal of the 35 U.S.C. § 112, first paragraph "written description", rejection of claims 48-50, 52-56 and 65-68 is requested.

The claims are submitted to be in condition for allowance and Reversal of the Final Rejection is requested.

Respectfully submitted,

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(8) CLAIMS APPENDIX

48. An isolated protein or glycoprotein inhibitor of xylanase, which inhibitor is a water-soluble, alkaline protein or glycoprotein, which protein or glycoprotein comprises an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1, said inhibitor having a pI of greater than about 7.0, and a molecular weight of about 40-43 kDa as measured by SDS-PAGE.

49. An isolated protein or glycoprotein inhibitor of xylanase, which inhibitor is a water-soluble, alkaline protein or glycoprotein, which protein or glycoprotein comprises an N-terminal amino acid sequence which is at least 70% homologous to SEQ ID NO:1, said inhibitor having a pI of greater than about 7.0 and a molecular weight of about 40-43 kDa as measured by SDS-PAGE, said inhibitor resolving as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol, said two separate bands having molecular weights of about 30 kDa and about 10 kDa.

50. The isolated protein or glycoprotein inhibitor of claim 48 wherein said protein or glycoprotein comprises an amino acid sequence of SEQ ID NO:1.

52. The isolated protein or glycoprotein inhibitor of claim 48 wherein said inhibitor is obtainable from a cereal plant, or cereal plant fraction thereof.

53. The isolated protein or glycoprotein inhibitor of claim 49 wherein said inhibitor is obtainable from a cereal plant, or cereal plant fraction thereof.

54. The isolated protein or glycoprotein inhibitor of claim 48 wherein said inhibitor is obtainable from a plant, or cereal plant fraction thereof, selected from the group consisting of wheat, rye, triticale, barley, sorghum, oats, maize and rice.

55. The isolated protein or glycoprotein inhibitor of claim 49 wherein said inhibitor is obtainable from a plant, or cereal plant fraction thereof, selected from the group consisting of wheat, rye, triticale, barley, sorghum, oats, maize and rice.

56. The isolated protein or glycoprotein inhibitor of claim 50 wherein said inhibitor is obtainable from a plant, or cereal plant fraction thereof, selected from the group consisting of wheat, rye, triticale, barley, sorghum, oats, maize and rice.

65. An isolated wheat protein or glycoprotein inhibitor of xylanase, which inhibitor is a water-soluble, alkaline protein or glycoprotein, which protein or glycoprotein has a pI of greater than about 7.0 and has a molecular weight of about 40-43 kDa as measured by SDS-PAGE, said protein or glycoprotein being able to resolve as two separate bands on SDS-PAGE after reduction with ß-

mercaptoethanol, said two separate bands having molecular weights of about 30 kDa and about 10 kDa.

66. An isolated cereal protein or glycoprotein inhibitor of xylanase, which inhibitor is a water-soluble, alkaline protein or glycoprotein, which protein or glycoprotein has a pI of greater than about 7.0 and has a molecular weight of about 40-43 kDa as measured by SDS-PAGE, said protein or glycoprotein being able to resolve as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol, said two separate bands having molecular weights of about 30 kDa and about 10 kDa, and wherein said cereal is selected from the group consisting of wheat, rye triticale, barley, sorghum, oats, maize and rice.

67. An isolated cereal protein or glycoprotein inhibitor of xylanase, which inhibitor is a water-soluble, alkaline protein or glycoprotein, which protein or glycoprotein has a pI of greater than about 7.0 and has a molecular weight of about 40-43 kDa as measured by SDS-PAGE, said protein or glycoprotein being able to resolve as two separate bands on SDS-PAGE after reduction with ß-mercaptoethanol, said two separate bands having molecular weights of about 30 kDa and about 10 kDa, and wherein said cereal is selected from the group consisting of wheat, rye and barley.

68. An isolated cereal proteinic or glycoproteinic inhibitor of xylanase, which inhibitor is a water-soluble, alkaline protein or glycoprotein, which protein or glycoprotein has a pI of greater than about 7.0 and has a molecular weight of 40-43 kDa as measured by SDS-PAGE and wherein when said cereal proteinic or glycoproteinic inhibitor of xylanase is a wheat proteinic or glycoproteinic inhibitor of xylanase said protein or glycoprotein is able to resolve as two separate bands on SDS-PAGE after reduction with β-mercaptoethanol, said two separate bands having molecular weights of about 30 kDa and about 10 kDa.

(9) EVIDENCE APPENDIX

Attached:

(a) WO01/98474.

Entered in record by appellants with Amendment of June 26, 2003 and listed on the PTO 1449 Form filed June 26, 2003, and considered by Examiner as indicated on page 2 of the Office Action dated November 18, 2004.

(b) List of 500 of 892 U.S. patents issued from 1976 to a recent search date which include the words BESTFIT and protein.

Entered in record by appellants as Appendix A to the Amendment filed August 29, 2003, and considered by the Examiner as indicated on page 2 of the Office Action dated November 17, 2003.

(c) Description of BESTFIT

Entered in record by appellants as Appendix B to the Amendment filed August 29, 2003, and considered by the Examiner as indicated on page 2 of the Office Action dated November 17, 2003.

(d) List of 27 granted U.S. Patents and representative claims wherein "percent homology" and/or BESTFIT are recited.

Entered in record by appellants as Appendix C to the Amendment filed August 29, 2003, and considered by the Examiner as indicated on page 2 of the Office Action dated November 17, 2003.

(e) Copy of 66 FR 1099, Friday, January 5, 2001

Entered in record by appellants as Appendix D to the Amendment filed August 29, 2003, and considered by the Examiner as indicated on page 2 of the Office Action dated November 17, 2003.

(f) *Eli Lilly v. University of California,* 43 USPQ 2d 1308 (Fed. Cir. 1997).

Entered in record as discussed by appellants in Amendment filed August 29, 2003, and considered by the Examiner as indicated on page 2 of the Office Action dated November 17, 2003.

(g) Press Release #00-15, USPTO, March 1, 2000.

Entered in record by appellants as Appendix E to the Amendment filed August 29, 2003, and considered by the Examiner as indicated on page 2 of the Office Action dated November 17, 2003.

(9) RELATED PROCEEDINGS APPENDIX

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WO 01/98474 A1

(54) Title: BIOCATALYST INHIBITORS

(57) Abstract: The present invention concerns a method for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or beta-glucanolytic enzymes, inhibitors obtainable by said method, and process for obtaining micro-organism, plant or plant material wherein the activity of the inhibitor according to the invention is increased or reduced and to the use of the inhibitor, using the cited micro-organism, plant or plant material and/or the use of endoxylanases selected or modified using these inhibitors in a variety of process and applications.

BIOCATALYST INHIBITORS

Field of the invention

This invention relates to a method for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8)(also referred to as xylanase), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes, which are present in micro-organisms, plants, plant materials or fractions thereof, (such as cereals, cereal grains, cereal flours or fractions thereof). The method comprises the use of two or more enzymes, especially endoxylanases, during the screening for inhibition activity. In a preferred embodiment, one of the endoxylanases is from *Bacillus subtilis*.

This invention also relates to a method for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC:3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading

enzymes such as endoxylanase (such as EC: 3.2.1.8)(also referred to as xylanase), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes, which are present in micro-organisms, plants, plant materials or fractions thereof, (such as cereals, cereal grains, cereal flours or fractions thereof). The method comprises an affinity chromatographic step with immobilised enzymes, especially endoxylanases, and/or antibodies against the said inhibitors, especially antibodies against an endoxylanase inhibitor. In a preferred embodiment, the immobilised endoxylanases are those from *Bacillus subtilis* and/or *Aspergillus niger*.

The present invention is also related to inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes, obtainable by said methods, as well as to feed or food compositions comprising said inhibitors and to the use of said inhibitors for screening enzymes such as endoxylanases that are totally, more, less or not inhibited by said inhibitors or for modifying enzymes, such as endoxylanases in such way that they are totally, more, less or not inhibited by said inhibitors, as well as to the use of said inhibitors in different areas of food, feed and/or beverage technologies, such as malting and brewing, the production of

animal feedstuffs such as to increase their conversion, the production of refrigerated and/or frozen doughs, such as to reduce syruping, the production of baked and/or extruded cereal products such as straight dough, sponge and dough (all said dough compositions comprising flour and water) and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles, the production of starch derived syrups, sorbitol, xylose and/or xylitol, the wheat gluten-starch separation industry, maize processing, the improvement of plant disease resistance, in nutraceutical or pharmaceutical applications such as maintaining the structure of dietary fiber material, and in the field of paper and pulp technologies.

The present invention also relates to polynucleotide sequences encoding inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes and to said inhibitors obtainable by a recombinant production process using said polynucleotide sequences encoding the recombinant inhibitors. The invention also relates to feed or food compositions comprising said recombinant inhibitors and the use of said recombinant inhibitors for screening enzymes such as endoxylanases that are totally, more, less or not inhibited by said inhibitors or for modifying enzymes, such as endoxylanases in such way that they are totally, more, less or not inhibited by said inhibitors, as well as to the use of said recombinant inhibitors in different areas of

food, feed and/or beverage technologies, such as malting and brewing, the production of animal feedstuffs such as to increase their conversion, the production of refrigerated and/or frozen doughs, such as to

reduce syruping, the production of baked and/or extruded cereal products such as straight dough, sponge and dough (all said dough compositions comprising flour and water) and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles, the production of starch derived syrups, sorbitol, xylose and/or xylitol, the wheat gluten-starch separation industry, maize processing, the improvement of plant disease resistance, in nutraceutical or pharmaceutical applications such as maintaining the structure of dietary fiber material, and in the field of paper and pulp technologies.

Background of the invention

Cereal grains contain three groups of important biopolymers: starch, proteins and non-starch polysaccharides. Starch and a large part of the protein fraction are located in the endosperm and serve as reserve material for the plant during germination and the initial stages of growth. They are degraded by amylases and proteases respectively [1]. The non-starch polysaccharides include mainly arabinoxylan (AX) and β-glucan which are part of the cell walls and are hydrolysed by xylanolytic and β-glucanolytic enzymes respectively [1, 2]. The degradation of these cell wall polysaccharides in the endosperm and aleurone layer during the germination improves the accessibility of starch and protein for amylases and proteases [3, 4]. Proteins that inhibit amylases [5-9] and proteases [10-13] have already been purified from cereals and have been characterised extensively. They possibly regulate the plant starch and nitrogen metabolism and/or play an important role in plant defence by inhibiting enzymic hydrolysis by micro-organisms and predators.

Recently, a new class of enzyme inhibitors, i.e. proteinaceous inhibitors of endo-β-1,4-xylanases (endoxylanases, EC: 3.2.1.8), has been discovered in cereals by Debyser and Delcour [14] and Debyser et al. [15]. Inhibition activity against such xylanolytic enzymes was found in different cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) [14, 16].

An endoxylanase inhibitor, named TAXI (*T. aestivum* L. endoxylanase inhibitor), was purified from wheat flour and characterised by Debyser and Delcour [14] and Debyser et al. [17] wherein for the screening of the inhibition activity a single endoxylanase from *Aspergillus niger* was used. TAXI has a molecular mass of ca. 40.0 kDa and occurs in two molecular forms A and B, B presumably as a result of proteolytic

modification of A [14, 16, 17]. As a result of reduction with β-mercaptoethanol, the modified molecular form B dissociates in two fragments with molecular masses of ca. 10.0 and 30.0 kDa respectively, whereas the molecular mass of the non-modified form does not change upon reduction. The inhibitor is heat sensitive and has a pI of ca. 8.8 [14, 16, 17]. Rouau and Surget [18] also found evidence for the presence of endoxylanase inhibitors in regular and durum wheats. These authors detected high inhibition activity against microbial endoxylanases in both wheat flour and bran. McLauchlan et al. [19] and Hessing and Happe [20] purified a wheat endoxylanase inhibitor structurally quite different from TAXI. In the isolation procedure [19], an endoxylanase, partially purified from a commercial *A. niger* hemicellulase preparation was used for the screening of the inhibition activity. The resulting inhibitor is monomeric, glycosylated and a heat sensitive protein. It has a pI of 8.7-8.9 [19] or higher than 9 [20], a molecular mass of 29.0 kDa [19] and 31.0 kDa [20] and was found to be a competitive inhibitor. The N-terminal amino acid sequence is 87 % identical with a sequence close to the N-terminus of the rice chitinase III polypeptide chain and shows no homology with the amino acid sequences of TAXI [19,20].

Further information on TAXI can be found in Sibbesen and Sørensen [36].

Summary of the invention

The present invention concerns methods for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes, preferably inhibitors of endoxylanase, of β-glucanase, of β-xylosidase, of α-L-arabinofuranosidase, and of other xylan, arabinoxylan and β-glucan degrading enzymes preferably obtained from micro-organisms, plants, plant materials or fractions thereof (such as cereals, cereal grains, cereal germs or fractions thereof, cereal flours or fractions thereof) by the use of two or more enzymes, especially endoxylanases, during the screening for endoxylanase inhibiting activity and/or by the application of affinity chromatography with immobilised enzymes, especially endoxylanases.

The inhibitory effect towards xylan and/or arabinoxylan hydrolysing enzymes can be e.g. demonstrated by the endoxylanase method with AZCL arabinoxylan (cfr. infra). Likewise, the inhibitory effect towards β-glucan hydrolysing enzymes can be e.g. demonstrated by the β-glucanase method with AZCL β-glucan (cfr. infra).

The present invention also concerns novel inhibitors obtainable by said purification methods, of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC:3.2.1.4), pentosanases and/or hemicellulases) especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes.

In this text, "An inhibitor of an enzyme" means

a molecule which is able to inhibit partially or totally the activity of said enzyme. In irreversible inhibition, the inhibitor is covalently linked to the enzyme or bound so tightly that its dissociation from the enzyme is very slow. In contrast, reversible inhibition may be characterised by a rapid equilibrium between the enzyme and the inhibitor. A competitive inhibitor blocks the active site and in this way prevents the substrate/active site interaction. As a consequence, the reaction rate is diminished. In the case of competitive inhibition, the inhibitor in many cases mimicks the normal substrate of said enzyme. For this type of inhibition, the Dixon plots (inverse of reaction rate, 1/V, versus inhibitor concentration, [I]) corresponding to the different substrate concentrations and the Lineweaver-Burk plots (inverse of reaction rate, 1/V, versus inverse of substrate concentration, 1/[S]) corresponding to different inhibitor concentrations intersect in the left quadrant and the vertical axis respectively. For non-competitive inhibition, both inhibitor and arabinoxylan can bind to the enzyme and this independent of the binding order. The inhibition can in most cases be explained by a change in conformation of the enzyme at or near the active site with a decreased turnover number as a result. In the case of non-competitive inhibition, however, the Dixon and Lineweaver-Burk plots intersect on the horizontal axis in the left quadrant. Competitive inhibition can be distinguished from non-competitive inhibition by determining whether the inhibition can be overcome by raising the substrate concentration. Inhibitors isolated from a specific biological species and that are of proteinaceous or glycoproteinaceous nature can be active against enzymes of the same species (i.e. endogenous enzymes) and/or against enzymes of different species (i.e. exogenous enzymes).

Advantageously, the inhibitors of the invention can be produced by micro-organisms or may be present in

various extraction media from micro-organisms or plant material, such as cereals or fractions thereof, such as cereal grains or fractions thereof, such as cereal germs or fractions thereof, such as cereal flours or fractions thereof, such as from wheat, durum wheat, rye, triticale, barley, sorghum, oats, maize and/or rice, from which they can be purified by the methods well known by the man skilled in the art. According to a preferred embodiment of the present invention, inhibitors are endoxylanase inhibitors which are typically water-soluble alkaline proteinaceous species, having a pI (i.e. -log of the isoelectric point) of greater than about 7.0. The endoxylanase inhibitor molecular weights as determined by SDS-page are typically 40-43 kDa. Following reduction with β-mercaptoethanol three SDS-page protein bands are found with SDS-page molecular weights of ca. 40-43 kDa, ca. 30 kDa, and ca. 10 kDa. The N-terminal sequences of the 40-43 kDa proteins or glycoproteins are typically as follows: SEQ ID No. 1 (TAXI I): Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Asp-Pro-Ala-Thr-Ser-Leu-Tyr-Thr-Ile-Pro-Phe-Xaa-Asp-Xaa-Ala, wherein the first Xaa being preferably Leu and wherein the second Xaa being preferably Leu; SEQ ID No. 2 (TAXI II): Lys-Gly-Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Asp-Thr-Ala-Thr-Ser-Leu-Tyr-Thr-Ile-Pro-Phe or SEQ ID No. 3 (HvXI): Lys-Ala-Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Asp-Ala-Ala-Thr-Ser-Leu-Tyr-Thr-Ile-Xaa-Xaa, wherein the first Xaa being preferably Pro and wherein the second Xaa being preferably Phe. The 30 kDa band has the above described typical N-terminal amino acid SEQ ID No.1 or SEQ ID No.2 or SEQ ID No.3, while the N-terminal amino acid sequence of the 10 kDa band is typically as follows: SEQ ID No. 4 (TAXI I): Gly-Ala-Pro-Val-Ala-Arg-Ala-Val-Glu-Ala-Val-Ala-Pro-Phe-Gly-Val-Xaa-Tyr-Asp-Thr, wherein Xaa being preferably Leu; or SEQ ID No. 5 (TAXI II): Gly-Ala-Pro-Val-Ala-Arg-Ala-Val-Ile-Pro-Val-Ala-Pro-Phe-Glu-Leu-Xaa-Tyr-Xaa-Thr-Lys-Ser-Leu-Gly-Asn, wherein the first Xaa being preferably Leu and wherein the second Xaa being

preferably Asp; or SEQ ID No. 6 (HvXI): Gly-Ala-Leu-Ala-Ala-Xaa-Gly-Val-Asn-Pro-Val-Ala-Pro-Phe-Gly-Xaa-Xaa-Tyr-Asp-Ala-Xaa-Thr-Xaa-Xaa, wherein the first Xaa is unknown, the second Xaa being preferably Leu, the third Xaa is unknown, the fourth Xaa is unknown, the fifth Xaa being preferably Asn, and wherein the sixth Xaa being preferably Gly.

Therefore, the present invention is also related to an inhibitor with a SDS-page molecular weight of typically 40-43 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 1. or SEQ ID No.2 or SEQ ID No.3

The present invention is furthermore also related to an inhibitor with a SDS-page molecular weight of typically 30 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 1. or SEQ ID No.2 or SEQ ID No.3.

The present invention is furthermore also related to an inhibitor with a SDS-page molecular weight of typically 10 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 4. or SEQ ID No.5 or SEQ ID No.6.

Advantageously, said markers are the end-terminal amino acid sequences of the protein or glycoprotein.

According to the invention, a marker of a protein or glycoprotein means a specific amino acid sequence (or its corresponding nucleotide sequence) that is able to distinguish one protein family from another protein family.

Another aspect of the invention involves the corresponding polynucleotide sequences of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases) especially inhibitors of

pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes.

The invention concerns isolated polynucleotides that encode for endoxylanase inhibitors from wheat, rye, rice, maize, oat and barley.

The invention also relates to amino acid sequences of endoxylanase inhibitors from wheat comprising the complement of SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions with SEQ ID No. 10, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 and SEQ ID No. 39.

The invention also relates to amino acid sequences of an endoxylanase inhibitor from barley comprising the complement of SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions with SEQ ID No. 14.

The invention also relates to amino acid sequences of endoxylanase inhibitors from rye, rice, maize and oat comprising the complement of SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 41, SEQ ID No. 43 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions with SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 40 and SEQ ID No. 42.

Thus the invention additionally features nucleic acid sequences encoding polypeptides, oligonucleotides, peptide nucleic acids (PNA), fragments, portions or antisense molecules thereof, and expression

vectors and host cells comprising polynucleotides that encode endoxylanase inhibitors such as TAXI I.

The invention also relates to a method for obtaining said inhibitors from a micro-organism, such as a genetically modified micro-organism which expresses said inhibitors, from a plant, or from a plant material (such as cereals, cereal grains, cereal germs or fractions thereof, cereal flours or fractions thereof, by subjecting said plant, said plant material and/or said micro-organism to one or more extraction and/or fractionation steps).

Another aspect of the present invention is related to a method for genetically transforming a micro-organism, a plant or a plant material in order to obtain the expression of the inhibitors according to the invention wherein the micro-organism, the plant or plant material is genetically modified by the introduction of a genetic material encoding said inhibitors into the micro-organism, the plant or plant material and obtain their translation and expression by genetic engineering methods well known by the man skilled in the art.

The invention furthermore relates to processes aiming at changing, preferably reducing or increasing levels of said inhibitors in a micro-organism, a plant or a plant material, by reducing or increasing the expression of said inhibitors, by the methods well known by the man skilled in the art and/or by using molecules which are able to block the inhibitor activity or activate said inhibitor.

The invention furthermore relates to use of said inhibitors for screening enzymes, such as endoxylanases that are totally, more, less or not inhibited by said inhihibitors or for modifying these enzymes, such as endoxylanases, by the methods well known by the man skilled in the art, in such way that they are totally, more, less or not inhibited by said inhibitors.

The invention furthermore relates to the obtained inhibitors, micro-organisms, plants, plant materials, and/or fractions thereof and to their use in different areas of food, feed and/or beverage technologies, such as improving malting and brewing, improving animal feedstuffs efficiency, baked and/or extruded cereal products (such as straight dough, sponge and dough and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles), improving the production of refrigerated and/or frozen doughs, such as to reduce syruping, (all said dough compositions comprising water and flour), improving the production of starch derived syrups, sorbitol, xylose and/or xylitol, improving wheat gluten-starch separation and production, maize processing, improving plant disease resistance, improving nutraceutical or pharmaceutical applications (such as maintaining the structure of dietary fiber material), and improving paper and pulp technologies.

The present invention will be described in details in the following description of a preferred embodiment without limiting the scope of the present invention.

Detailed description of the invention

The inventors unexpectedly found that, using an endoxylanase, such as the *Bacillus subtilis* endoxylanase, on top of the previously described *A. niger* endoxylanase for screening the inhibition activity during the isolation process of TAXI [14], the endoxylanase inhibitor (TAXI), as described by Debyser and Delcour [14] and Debyser et al. [17], is in fact a mixture of at least two endoxylanase inhibitors, i.e. TAXI I and TAXI II. Both inhibitors unexpectedly were shown to have comparable molecular masses and structures but they clearly differ from one another in pI

and endoxylanase specificity. In this way, a combination of two or more endoxylanases may be used for the isolation of endoxylanase inhibitors with a varying selectivity towards endoxylanases. It follows that the use of more endoxylanases can facilitate the identification and/or purification of inhibitors in mixtures of endoxylanase inhibitors.

The present invention for the first time shows that, on the one hand, wheat contains at least two types of TAXI-like endoxylanase inhibitors that differ in their endoxylanase specificity and that, on the other hand, at least one such inhibitor occurs in barley.

We unexpectedly found that, depending on the endoxylanase used for studying the type of inhibition, either a competitive or a non-competitive type of inhibition can be observed.

Furthermore, we describe for the first time a method for the purification of endoxylanase inhibitors, comprising of an affinity chromatographic step with immobilised endoxylanases.

We also document for the first time a new technique for the purification of endoxylanases from commercially available enzyme preparations based on affinity chromatography with an immobilised cocktail of 'TAXI'-like endoxylanase-inhibitors.

DNA sequences coding for endoxylanase inhibitors or part thereof are determined. For the first time, a recombinant active endoxylanase inhibitor from wheat was produced by a micro-organism.

DEFINITIONS

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, recombinant DNA (e.g. DNA prepared by use of recombinant DNA techniques), synthetic DNA, and RNA, as well as combinations thereof. Preferably, the term "nucleotide sequence" means DNA. The nucleotide sequences of the present invention may be single or double stranded. The nucleotide sequences of the present invention may include within them synthetic or modified nucleotides. A number of different types of modifications to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5'ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out to enhance the in vivo activity or life span of nucleotide sequences of the present invention.

The terms "variant" or "homologue" with respect to the nucleotide sequence of the present invention and the amino acid sequence of the present invention are synonymous with allelic variations of the sequences.

In particular, the term "homology" as used herein may be equated with the term "identity".

Furthermore, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of present invention, which will be limited only by the appending claims.

Examples

In what follows, the purification and partial characterisation of two endoxylanase inhibitors from wheat(*Triticum aestivum* L., var. Soissons), TAXI I and TAXI II (Example 1), and one endoxylanase inhibitor from barley (*Hordeum vulgare* L., var. Hiro), HvXI (Example 2), will be examined. In this context, an approach to isolate the inhibitors using cation exchange and gel filtration chromatography as the main techniques will be described. Furthermore, the isolation of ('TAXI'-like) endoxylanase inhibitors from a commercial wheat flour (likely a mixture of different wheat varieties), rye flour and barley whole meal using an alternative approach, i.e. affinity chromatography with immobilised endoxylanase will be discussed (Example 3). Furthermore, a new method based on affinity chromatography with immobilised 'TAXI'-like endoxylanase inhibitors to isolate endoxylanases from commercially available enzyme preparations will be examplified (example 4). We also describe corresponding DNA sequences (Examples 5,6,7,8,9 and 10) and recombinant expression of endoxylanase inhibitors (Example 11).

General experimental methods for examples 1 and 2

Materials

All reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless specified otherwise.

Endoxylanase (EC: 3.2.1.8) M1 from *Trichoderma viride* (family 11), endoxylanase M4 and α-L-arabinofuranosidase (arabinofuranosidase, EC: 3.2.1.55) from *Aspergillus niger* (family 11), endoxylanase M6 from a rumen microorganism culture filtrate, azurine-crosslinked wheat AX

tablets (AZCL-AX) and soluble wheat AX (medium viscosity) were from Megazyme (Bray, Ireland). Endoxylanases from *Bacillus subtilis* (family 11) and *Aspergillus aculeatus* (family 10) were from NV Puratos (Groot-Bijgaarden, Belgium). A β-D-xylosidase (xylosidase, EC 3.2.1.37) from *A. niger* and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Bornem, Belgium). The digoxigenin (DIG) glycan detection kit® was from Boehringer (Mannheim, Germany).

All electrophoresis media and markers, chromatographic media and nitrocellulose blot membranes were from Pharmacia Biotech (Uppsala, Sweden).

Wheat (*Triticum aestivum* L., var. Soissons) and barley (*Hordeum vulgare* L., var. Hiro), were from AVEVE (Landen, Belgium) and were milled with a Bühler MLU-202 mill (Uzwil, Switzerland) and a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden) respectively.

Protein determination

Protein concentrations were determined according to the Coomassie Brilliant Blue method of Bradford [21] with BSA as a standard.

Endoxylanase inhibition assay procedure

The inhibition activities of a set of samples were determined with the Xylazyme-AX method as described by Debyser [16]. Solutions of the *T. viride*, the *A. niger*, the *A. aculeatus* and the *B. subtilis* endoxylanases were prepared in sodium acetate buffer (25.0 mM, pH 5.0) with BSA (0.5 mg/ml) whereas the solution of the rumen microorganism culture filtrate was prepared in sodium phosphate buffer (25 mM, pH 6.0) with BSA (0.5 mg/ml). All endoxylanase solutions contained 2.0 enzyme units per 1.0 ml. One enzyme unit corresponds to an increase in extinction at 590 nm, using the xylazyme-AX method (cfr. infra), of 1.0.

Endoxylanase solution (0.5 ml) was preincubated

for 30 min at room temperature with an equal amount of sample (same buffer as enzyme solution), possibly containing inhibition activity. The mixtures were kept at 30 °C and after 10 min an AZCL-AX tablet was added. Next, they were incubated for 60 min at 30 °C. The reaction was terminated by adding 1.0% (w/v) tris-hydroxymethylaminomethane (Tris) solution (10.0 ml) and vigorous vortex stirring. After 10 min at room temperature, the tubes were shaken vigorously and the contents filtered through a Schleicher & Schuell filter (ϕ 90 mm) (Dassel, Germany). The absorbances at 590 nm (A_{590}) were measured against a control, prepared by incubating the sample with buffer instead of enzyme solution, with an Ultraspec III® UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The difference between the absorbance values of samples and another control, prepared by using buffer instead of sample, is a measure for the inhibition activity, expressed as percent reduction of endoxylanase activity.

Arabinofuranosidase and xylosidase inhibition assay procedure

The method used was based on that by Cleemput et al. [22]. *p*-Nitrophenyl arabinose and *p*-nitrophenyl xylose were used as substrates for measuring the α-L-arabinofuranosidase and β-D-xylosidase activities respectively, in the presence or absence of inhibitor. Substrate (0.05 mmol), arabinofuranosidase (50 µl), xylosidase (1.0 ml), TAXI I (234 µg), TAXI II (580 µg) and HVXI (460 µg) were separately dissolved in Mes buffer (50 mM, pH 5.5; 5 ml). Enzyme (25 µl) and endoxylanase inhibitor (25 µl) solutions were preincubated for 30 min at room temperature. Substrate (100 µl) was added and after 30 min at 30 °C the reaction was terminated by adding 1.0% (w/v) Tris solution (1.5 ml). Finally, the absorbance at 410 nm (A_{410}) was measured against a control.

Protein electrophoresis

SDS-PAGE under non-reducing and reducing conditions was performed on 20 % polyacrylamide gels with a PhastSystem® unit (Pharmacia Biotech, Uppsala, Sweden), according to the method of Laemmli [23]. β-Mercaptoethanol [5% (v/v)] was used as reducing agent. Low molecular weight (LMW) markers were α-lactalbumin (14.0 kDa); trypsin inhibitor (20.1 kDa); carbonic anhydrase (30.0 kDa); ovalbumin (43.0 kDa); albumin (67.0 kDa); phosphorylase b (94.0 kDa). The pI of the inhibitor was determined with the same instrument using polyacrylamide gels containing ampholytes (pH 3-9) and appropriate standards (Pharmacia Biotech calibration kit, pI 3.5-9.3). All gels were silver stained according to the instructions of the manufacturer (Pharmacia Biotech, Development Technique file N° 210).

Protein sequencing

TAXI I (25 µg), TAXI II (25 µg) and HVXI (25 µg) were sub-mitted to SDS-PAGE under reducing conditions (Laemmli, 1970) [23] in a SE 600 Series gel electrophoresis unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA). The slab gel (140.0 × 160.0 × 1.5 mm) consisted of a stacking gel [3.88% (w/v) T, 1.33% (w/v) C] and a running gel [17.57% (w/v) T, 0.46% (w/v) C]. Separation was achieved by using a current of 30 mA for 4 h at room temperature. The proteins were electroblotted onto a nitrocellulose membrane with the Trans-Blot® Semi-Dry Electroforetic Transfer Cell (Bio-Rad, Nazareth, Belgium), using an electric potential difference of 10 V for 1 h at room temperature, and were subjected to Edman degradation. The N-terminal amino acid sequences were determined with an Application Biosystems 477 A Protein Sequencer, connected on line with a 120 A phenylthiohydantoin-amino-acid analyser (Perkin Elmer, Lennik, Belgium).

Glycan detection

For glycan detection, the digoxigenin (DIG) glycan assay was carried out as described by Roels and Delcour [24]. TAXI I (1.0 mg/ml), TAXI II (1.0 mg/ml), HVXI (1.0 mg/ml), the positive control protein transferrin (1.0 mg/ml) and the negative control protein creatinase (1.0 mg/ml) were separated by SDS-PAGE under reducing conditions as described above, but using the sample buffer advised by the supplier of the DIG glycan detection kit®. The proteins were electroblotted onto a nitrocellulose membrane with a semi-dry PhastTransfer® unit (Pharmacia Biotech, Uppsala, Sweden), using an electric potential difference of 20 V for 30 min at 15 °C. On the blot, the vicinal diols of the glycans were converted to aldehydes with metaperiodate and labelled with the steroid hapten DIG via hydrazide. The labelled glycoconjugates were detected with a digoxigenin specific antibody conjugated to alkaline phosphatase. In the presence of the appropriate substrate, blue-purple bands appeared where the phosphatase was present. Oxidation, labelling and detection were performed according to the kit instructions (Method B).

Partial purification of wheat and barley endoxylanase inhibitors

The extraction of wheat flour and barley whole meal, the initial concentration and partial purification steps of the wheat and barley endoxylanase inhibitors were as described by Debyser and Delcour [14] and Debyser et al. [17].

Step I. Preparation of wheat flour or barley whole meal extracts

Wheat flour (10.0 kg) or barley whole meal (10.0 kg) were suspended in 0.1% (w/v) ascorbic acid (50.0 l), extracted over-night at 7 °C and centrifuged (10,000*g*; 30 min; 7 °C). To the supernatants, 2.0 g/l $CaCl_2$ was added and

the pH's were raised to 8.5 with 2.0 N NaOH to precipitate the pectins. The extracts were left overnight (7 °C) and centrifuged (10,000 *g*, 30 min, 7 °C). The pH's were adjusted to 5.0 with 2.0 M HCl.

Step II. Concentration and partial purification by cation exchange chromatography (CEC)

At pH 5.0, proteins with endoxylanase inhibiting activity from the wheat flour and barley whole meal extracts were retained by CEC on a SP Sepharose® Fast Flow column (90x90 mm). In both cases, the column was equilibrated with sodium acetate buffer (25 mM, pH 5.0; 500.0 ml) and a protein fraction was eluted with 0.5 M NaCl (1.0 l). The eluates were dialysed against deionised water (7 °C, 48 h) and lyophilised (= CEC_{wheat} material, 17.0 g and CEC_{barley} material, 10.8 g).

Example 1 Isolation and characterization of two xylanase inhibitors from wheat (TAXI I and TAXI II).

Further purification of wheat endoxylanase inhibitors

The wheat endoxylanase inhibitors, TAXI I and TAXI II, were further purified based on the method of Debyser and Delcour [14] and Debyser et al. [17]. After each purification step, the resulting fractions were assayed for endoxylanase inhibition activity with *A. niger* and *B. subtilis* endoxylanases and the purity was checked using SDS-PAGE.

Step 1. Purification by CEC

Batches of CEC_{wheat} material (4.0 g) in sodium acetate buffer (25 mM, pH 5.0; 400.0 ml) were applied on a SP Sepharose® Fast Flow column (26X300 mm), equilibrated with sodium acetate buffer (25 mM, pH 5.0; 200.0 ml). The proteins were eluted with a linear gradient of 0.0 to 0.5 M NaCl in 800.0 ml and a flow of 1.0 ml/min. Two separate fractions, one with high inhibition activity against *B. subtilis* and *A. niger* endoxylanases and one with high activity against *B. subtilis* endoxylanase but low activity against *A. niger* endoxylanase, were dialysed against deionized water (7 °C, 48 h) and lyophilised (= CEC_{wheat} I, 4.7 g, and CEC_{wheat} II, 2.9 g, respectively).

Step 2. Purification by gel permeation chromatography (GPC)

Batches of CEC_{wheat} I (20 mg) and CEC_{wheat} II (20 mg) in sodium acetate buffer (25 mM, pH 5.0; 1.0 ml) were fractionated by GPC on a Hiprep® Sephacryl® S-100 column (26X670 mm) with the same buffer (400 ml) and a flow of 0.7 ml/min. The active fractions were pooled (= GPC_{wheat} I, 590 mg in 2500 ml, and GPC_{wheat} II, 320 mg in 1630 ml, respectively).

Step 3. Purification by CEC

GPC_{wheat} I and GPC_{wheat} II were diluted three times. Batches of the diluted GPC_{wheat} I (100.0 ml) and diluted GPC_{wheat} II (100.0 ml) were fractionated by CEC on a MonoS® HR 5/5 column (5×50 mm), equilibrated with sodium acetate buffer (25 mM, pH 4.0; 5.0 ml) and sodium phosphate buffer (20 mM, pH 6.5; 5.0 ml) respectively. The bound proteins were eluted with a linear gradient of 0.0 to 0.6 M NaCl in 60.0 ml and a flow of 1.0 ml/min. The fraction, as a result of fractionation of GPC_{wheat} I and containing inhibition activity against *B. subtilis* and *A. niger* endoxylanases, and the fraction, which resulted from fractionation of GPC_{wheat} II and had activity against *B. subtilis* endoxylanase but not against *A. niger* endoxylanase, were used for further purification of TAXI I and TAXI II respectively. They were diluted three times, acidified to pH 4.0 with 1.0 N acetic acid and chromatographed again on the same MonoS® column, equilibrated with sodium acetate buffer (25 mM, pH 4.0). The same flow and salt gradient were used. We finally obtained 12.0 mg TAXI I and 9.5 mg of TAXI II.

Inhibition type determination

For TAXI I, the inhibition kinetics was studied with the *A. niger* (Megazyme, Bray, Ireland) and the *B. subtilis* (Puratos, Groot-Bijgaarden, Belgium) endoxylanase and for TAXI II only with the *B. subtilis* endoxylanase, because of its lack of inhibition activity against the *A. niger* endoxylanase, as discussed earlier. In all cases, soluble wheat arabinoxylan was used as a substrate. For each of the inhibitor/enzyme combinations the reaction rates for different substrate and inhibitor concentrations were measured. They resulted in the corresponding Dixon and Lineweaver-Burk plots.

For determining the reaction rates, a modified Somogyi reducing sugar assay was used [25]. For this method, the following reagents were prepared: reagent A (25.0 g anhydrous sodium carbonate, 25.0 g sodium potassium tartrate and 200.0 g anhydrous sodium sulphate in 1.0 l demineralised water), reagent B (30.0 g copper sulphate pentahydrate and 4 drops concentrated sulphuric acid in 200.0 ml demineralised water), reagent C (50.0 g ammonium molybdate dissolved in 900 ml demineralised water, 42.0 ml concentrated sulphuric acid and 6.0 g sodium arsenate heptahydrate dissolved separately in 50.0 ml demineralised water were mixed and the total volume was adjusted to 1.0 l), reagent D (1.0 ml of reagent B and 25.0 ml reagent A) and reagent E (one part reagent C and four parts demineralised water).

Wheat arabinoxylan (50.0, 33.3, 25.0, 20.0, 16.6, 14.2, 12.5 and 11.1 mg) was dissolved in sodium acetate buffer (100 mM, pH 5.0; 10.0 ml). The endoxylanase and endoxylanase inhibitor solutions were prepared in the same buffer containing BSA (0.5 mg/ml). The latter solutions contained 0.0 to 11.0 µg/ml TAXI I or TAXI II. The endoxylanases were diluted to such an extent that in the above described Somogyi reducing sugar assay, carried out with 0.5 % (w/w) soluble wheat AX and in absence of endoxylanase inhibitor, an increase in extinction comparable to that of the standard curve solution with the highest xylose concentration was obtained (cfr. infra).

Wheat arabinoxylan solution (0.5 ml) was mixed with 0.1 ml sodium acetate buffer (100 mM, pH 5.0) containing BSA (0.5 mg/ml) or with 0.1 ml endoxylanase inhibitor solution and incubated at 30 °C. After 10 min endoxylanase (0.1 ml), equilibrated at the same temperature, was added. The reaction was terminated 15 min later by adding reagent D (0.5 ml), after which all tubes were boiled for 20 min. The samples were cooled at room temperature and mixed with reagent E (3.0 ml) for colour development. After 15 min the

absorbance was measured at 520 nm against a reagent blank. For the latter, sodium acetate buffer (100 mM, pH 5.0) with BSA (0.5 mg/ml) was used instead of endoxylanase and endoxylanase inhibitor. To assess the reaction rates, a xylose standard curve was prepared by replacing endoxylanase and endoxylanase inhibitor with xylose solutions prepared in the same buffer (0-250.0 µg/ml).

Results

Inhibitor purification

Using the purification method described above, and *A. niger* and *B. subtilis* endoxylanases for assaying inhibition activity, TAXI I and TAXI II were purified to homogeneity from wheat flour. After initial fractionation by cation exchange chromatography (CEC) on SP Sepharose® Fast Flow columns, two protein fractions, one with high inhibition activity against *B. subtilis* and *A. niger* endoxylanases (CEC_{wheat} I) and one with high activity against *B. subtilis* endoxylanase but much lower activity against *A. niger* endoxylanase (CEC_{wheat} II), were obtained, indicating specificity of different inhibitors present. Figure 1 shows the SP Sepharose® Fast Flow chromatogram (–) of CEC_{wheat}-material, with indication of the NaCl-gradient (-) and the inhibition activities against *B. subtilis* (•) and *A. niger* (o) endoxylanases. CEC_{wheat} I and CEC_{wheat} II eluted at NaCl concentrations of 0.12 to 0.22 M and 0.23 to 0.27 M respectively. Both CEC_{wheat} I and CEC_{wheat} II contained no significant inhibition activity against *A. aculeatus* endoxylanase. The ratio of inhibition activity against *B. subtilis* endoxylanase to inhibition activity against *A. niger* endoxylanase ($IA^{B.s.}/IA^{A.n.}$) for diluted (×100) CEC_{wheat} I and CEC_{wheat} II was 1.11 and 3.21 respectively. This difference in $IA^{B.s.}/IA^{A.n.}$ indicated that we were dealing with mixtures of

two endoxylanase inhibitors, further referred to as TAXI I and TAXI II.

From CEC_{wheat} I, TAXI I was purified by gel permeation chromatography (GPC) on a Hiprep® Sephacryl® S-100 column, at which it eluted at a volume of 127.5 to 138.5 ml (GPC_{wheat} I), followed by CEC on a MonoS® column at pH 4.0, at which it eluted at NaCl concentrations of 0.27 to 0.36 M. GPC_{wheat} I contained also TAXI II but at much lower levels than TAXI I. With CEC on MonoS®, TAXI II, characterised by a much higher $IA^{B.s.}/IA^{A.n.}$ than TAXI I (cfr. infra), resulted in an additional but smaller inhibition activity peak in the chromatogram. The final purification step was performed twice at the same pH in order to increase the purity of TAXI I. Figure 2A displays the chromatogram (–) of the final separation of TAXI I on MonoS® with indication of the NaCl-gradient (-).

TAXI II was isolated from CEC_{wheat} II in a similar way, but CEC on MonoS® was performed first at pH 6.5 and secondly at pH 4.0. CEC_{wheat} II contained, in contrast to CEC_{wheat} I, much more TAXI II than TAXI I. With GPC, TAXI II eluted at the same volume as TAXI I (GPC_{wheat} II) and with CEC on MonoS® at pH 6.5 and 4.0, TAXI II eluted at NaCl concentrations of 0.08 to 0.11 M and 0.42 to 0.49 M respectively. In analogy with the above, a small additional activity peak, caused by the presence of TAXI I, was observed with CEC on MonoS® at pH 6.5. Figure 2B displays the MonoS® CEC chromatogram (–) of almost pure TAXI II, separated at pH 4.0, with indication of the NaCl-gradient (-).

Inhibitor partial molecular characterisation

Figure 3 shows the SDS-PAGE profiles of TAXI I (A) and TAXI II (B) with in lane 1 the low molecular mass markers (the size of the markers indicated on the left), in lane 2 pure inhibitor under reducing conditions and in lane 3

pure inhibitor under non-reducing conditions. The profiles (non-reducing conditions) of purified TAXI I and TAXI II show two polypeptides of ca. 40.0 kDa. Under reducing conditions, additional 30.0 and 10.0 kDa polypeptides can be seen. These findings are in agreement with those of Debyser and Delcour [14] and Debyser et al. [17]. The pI of TAXI II is at least ca. 9.3 and is therefore higher than that of TAXI I, which has a pI of ca. 8.8.

The 30.0 and 40.0 kDa polypeptides have the same N-terminal amino acid sequences, which for TAXI I and TAXI II are : SEQ ID No. 1 and SEQ ID No. 2 respectively. The N-terminal amino acid sequences of the TAXI I and TAXI II 10.0 kDa polypeptides are SEQ ID No. 4 and SEQ ID No. 5 respectively. These data confirm the molecular structure model of TAXI by Debyser and Delcour [14] and Debyser et al. [17]. Since the N-terminal sequences of the 30.0 and 40.0 kDa polypeptides are identical, the 10.0 and 30.0 kDa polypeptides, held together by one or more disulfide bonds, are probably derived from the 40.0 kDa polypeptide by proteolytic modification.

TAXI I and TAXI II are not glycosylated, as evidenced from the DIG glycan detection kit® results. Even after 15 h of colour development, no bands appeared on the blot for both inhibitors. The positive and negative control proteins, transferrin and creatinase respectively, gave the expected results.

Inhibition activities against xylanolytic enzymes

Figures 4 and 5 show the activities of different levels of TAXI I and TAXI II respectively against five different endoxylanases, i.e. *A. aculeatus* (●), *A. niger* (■), *B. subtilis* (♦), *T. viride* (X) and rumen microorganism culture filtrate endoxylanases (▲). Except in the case of the *A. niger* endoxylanase, under the specified conditions TAXI I and TAXI II have similar inhibition activity profiles, which

are depicted in Figures 4 and 5.

TAXI I has high activities against the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanases, low activity against the rumen micro-organism endoxylanases and little if any activity against the *A. aculeatus* endoxylanase. The maxima of inhibition are slightly above 90 % for the first two endoxylanases, ca. 82 % for the *B. subtilis* endoxylanase and ca. 15 % for the rumen micro-organism endoxylanases. Under the test conditions, different levels of TAXI I (ca. 0.10, ca. 0.08 and ca. 0.20 µg respectively) reduce the activities of the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanase with 50 %.

TAXI II has high activities against the *T. viride* and the *B. subtilis* endoxylanase, low activity against the rumen micro-organism endoxylanases and little if any activity against the *A. niger* and the *A. aculeatus* endoxylanase. The maxima of inhibition are slightly above 90 % for the first endoxylanase, ca. 77 % for the *B. subtilis* endoxylanase and ca. 8 % for the rumen micro-organism endoxylanases. As for TAXI I, different quantities of TAXI II (ca. 0.07 and ca. 0.28 µg respectively) reduce the activities of the *T. viride* and the *B. subtilis* endoxylanase with 50 %.

Because after boiling (15 min, pH 5.0) no inhibition activity could be found against the mentioned endoxylanases, both inhibitors are heat sensitive.

Other xylanolytic enzymes, an arabinofuranosidase and a xylosidase from *A. niger*, were not inhibited by TAXI I and TAXI II.

Inhibition type

Figure 6 shows the Dixon plots corresponding to TAXI I and *B. subtilis* endoxylanase for substrate concentrations [S] = 5.00 (♦), 3.33 (■), 2.50 (▲), 2.00 (X), 1.67 (✱) and 1.43 (●), mg/ml wheat arabinoxylan.

Figure 7 shows the Lineweaver-Burk plots corresponding to TAXI I and *B. subtilis* endoxylanase for inhibitor quantities [I] = 0.0 (■), 0.22 (♦), 0.66 (▲) and 1.10 (×) μg

Figure 8 shows the Dixon plots corresponding to TAXI II and *B. subtilis* endoxylanase for substrate concentrations [S] = 5.00 (♦), 2.50 (▲), 1.67 (✱) and 1.25 (+) mg/ml wheat arabinoxylan.

Figure 9 shows the Lineweaver-Burk plots corresponding to TAXI II and *B. subtilis* endoxylanase for inhibitor quantities [I] = 0.0 (■), 0.49 (♦), 0.74 (▲) and 0.98 (×) μg

Figure 10 shows the Dixon plots corresponding to TAXI I and *A. niger* endoxylanase for substrate concentrations [S] = 5.00 (♦), 3.33 (●), 2.50 (▲) and 2.00 (■) mg/ml wheat arabinoxylan.

Figure 11 shows the Lineweaver-Burk plots corresponding to TAXI I and *A. niger* endoxylanase for inhibitor quantities [I] = 0.0 (■), 0.22 (♦), 0.66 (▲), 1.10 (×) μg

Depending on the endoxylanase, two different types of inhibition were observed. TAXI I and TAXI II both inhibited the *B. subtilis* endoxylanase in a non-competitive manner (Figures 6, 7, 8 and 9), where as the *A. niger* endoxylanase was inhibited competitively by TAXI I (Figures 10 and 11). For non-competitive inhibition, the Dixon plot (inverse of reaction rate, 1/V, versus inhibitor concentration, [I]) corresponding to the different substrate concentrations (Figures 6 and 8) and the Lineweaver-Burk plot (inverse of reaction rate, 1/V, versus inverse of substrate concentration, 1/[S]) corresponding to different inhibitor concentrations (Figures 7 and 9) intersect on the horizontal axis in the left quadrant. In the case of competitive inhibition, however, the curves intersect in the left

quadrant (Figure 10) and on the vertical axis (Figure 11) respectively.

Discussion

Two endoxylanase inhibitors (TAXI I and TAXI II) were purified from wheat and partially characterised. Both are non-glycosylated and have similar N-terminal amino acid sequences and SDS-PAGE profiles, indicating that there may be an evolutionary relationship between them. Their pI values are respectively ca. 8.8 and ca. 9.3 or higher. Except for the *A. niger* endoxylanase, TAXI I and TAXI II have, under the specified conditions, the same inhibition activity profiles, i.e. both inhibitors inhibited the *A. aculeatus*, *B. subtilis*, *T. viride* and rumen microorganism culture filtrate endoxylanases to a similar extent (Figures 4 and 5). In the case of the *A. niger* endoxylanase, however, TAXI I resulted in a strong inhibition where as for TAXI II little if any inhibition could be observed.

Using a BLAST (version 2.0.10) search [26] in public sequence databases, the N-terminal amino acid sequences of the 40.0 and 30.0 kDa polypeptides of TAXI I were found to be 66 % identical with internal sequences of an extracellular dermal glycoprotein precursor from *Arabidopsis thaliana* (amino acids 32-46: LLLPVTKDPSTLQYT) and a glucose-6-phosphate isomerase from *Escherichia coli* (amino acids 449-460: KDPATLDYVVPF) in a 15- and 12-amino acid overlap (amino acids 3-17 and 9-20 of TAXI I) respectively. The 40.0 and 30.0 kDa polypeptides of TAXI II are 64 % identical with an internal sequence of a ribulose-1,5-biphosphate carboxylase small subunit from *Fritillaria agrestis* (amino acids 38-51: PVTQKTATGLSTLP) in a 14-amino acid overlap (amino acids 8-21 of TAXI II).

The present results show that Debyser and Delcour [14] and Debyser et al. [17], in their reports on TAXI, probably studied a mixture of TAXI I and TAXI II.

Indeed, mixtures of these two proteins with the above mentioned inhibition specificities may very well have resulted in the observation that TAXI inhibited *B. subtilis* endoxylanase more effectively than the corresponding *A. niger* enzyme. Because the authors only screened with *A. niger* endoxylanase to purify TAXI, they probably picked up CEC_{wheat} I together with some material of CEC_{wheat} II with CEC on SP Sepharose® Fast Flow (Figure 1). Nevertheless, our observations are in line with the published [17] model for the molecular structure of the TAXI type endoxylanase inhibitors, maintaining that these proteinaceous inhibitors occur in two molecular forms A and B with a molecular mass of ca. 40.0 kDa. According to the model, following reduction with β-mercaptoethanol, form B dissociates in two fragments of ca. 10.0 and ca. 30.0 kDa, whereas the molecular mass of form A is not affected by the treatment. Since the N-terminal sequences of the ca. 30.0 and ca. 40.0 kDa polypeptides were identical, the ca. 10.0 and ca. 30.0 kDa polypeptides of form B, held together by one or more disulfide bounds, are probably derived from the ca. 40.0 kDa polypeptide (form A) by proteolytic modification. We have strong indications that form A is active as endoxylanase inhibitor, but to what extent form B is active, is not clear at present. It seems reasonable to assume that the first form (A) is a precursor of the second form (B) and that the inhibitor needs to be proteolytically modified to become more or less active. A mechanism where however a non-active protein is activated by proteolytic modification has been observed for an α-amylase inhibitor from bean (*Phaseolus vulgaris* L.) seeds [27, 28].

Studies on the inhibition type of TAXI I and TAXI II unexpectedly show that the type of inhibition depends on the endoxylanase used (Figures 6 to 11). The *A. niger* endoxylanase is inhibited by TAXI I by blocking the active site, i.e. TAXI I competes with arabinoxylan (competitive inhibition) and in the case of the *B. subtilis* endoxylanase,

both TAXI I or TAXI II and arabinoxylan can bind and this independent of the binding order, i.e. TAXI I and TAXI II do not compete with arabinoxylan (non-competitive inhibition). The last type of inhibition is however in contrast to the findings of Sørensen and Poulsen [29] and Mclauchlan et al. [19] who for endoxylanase inhibitors only observed competitive inhibition. The present unexpected finding probably can be explained by the choice of endoxylanase they used to study the inhibition kinetics.

The above described results and fig 5 demonstrate, that with the latter type of inhibition inhibitor/enzyme complexes can be formed that still have some residual activity. After all, for the *B. subtilis* endoxylanase and TAXI I or TAXI II the inhibition (non-competitive) as a function of inhibitor quantity reaches a maximum at about 80% inhibition, whereas for the *A. niger* endoxylanase and TAXI I (competitive inhibition) this is at about 95%. These residual endoxylanase activities can lead to different arabinoxylan degradation patterns and products than the endoxylanase activity in absence of TAXI I and TAXI II. This in turn implies that endoxylanase inhibitors can alter the functionality of certain endoxylanases, such as the *B. subtilis* endoxylanase, and/or can have an impact on the relative affinity and/or hydrolysis specificity and/or rate versus waterextractable and/or waterunextractable arabinoxylans.

In contrast to TAXI type endoxylanase inhibitors, the more recent inhibitor described by McLauchlan et al. [19] and Hessing and Happe [20] is monomeric and glycosylated and has a molecular mass of ca. 29.0 kDa. Its N-terminal amino acid sequence is 87% identical with a sequence of rice chitinase III in a 15-amino acid overlap and shows no homology with the reported amino acid sequences of either TAXI I or TAXI II. The monomeric endoxylanase inhibitor was found to inhibit an *A. niger* endoxylanase in a competitive

manner.

In wheat, endoxylanase inhibitors may have a dual function. They possibly play an important role in regulation of plant metabolism by inhibiting endogenous endoxylanases and/or in plant defence by inhibiting exogenous endoxylanases, produced by micro-organisms and predators. In contrast to exogenous endoxylanases, endogenous endoxylanases of wheat are less well documented apart from the work by Cleemput [30] and Cleemput et al. [22, 31] who purified two endoxylanases with different substrate specificities.

Example 2. Isolation and characterization of a xylanase inhibitor from barley (HVXI)

Further purification of a barley endoxylanase inhibitor

Barley endoxylanase inhibitor, HVXI, was further purified using a method identical to that for the further purification of TAXI II (cfr. supra). After each purification step, the resulting fractions were assayed for endoxylanase inhibition activity, both with *B. subtilis* and *A. niger* endoxylanases

Step 1. Purification by CEC

CEC_{barley} was separated in a way analogous to that of CEC_{wheat}, yielding fraction CEC_{barley}' (6.84 g).

Step 2. Purification by gel permeation chromatography (GPC)

CEC_{barley}' was separated in a way analogous to that of CEC_{wheat} I or CEC_{wheat} II, yielding fraction GPC_{barley} (640 mg in 3300 ml).

Step 3. Purification by CEC

HVXI was purified to homogeneity from GPC_{barley} much as TAXI II from GPC_{wheat} II, i.e. a first separation by CEC was performed on MonoS® at pH 6.5 followed by a second separation on the same column at pH 4.0. For both separations, the same gradient and flow were used. We finally obtained 18.0 mg HVXI.

Results

Inhibitor purification

By using an identical purification procedure as described for TAXI II, HVXI was purified from barley whole meal. Figure 12 shows the SP Sepharose® Fast Flow chromatogram

(–) of CEC_{barley}-material, with indication of the NaCl-gradient (-) and the inhibition activities against *A. niger* (o) and *B. subtilis* (•) endoxylanases. With CEC on SP Sepharose® Fast Flow the fractions with inhibition activity elute at NaCl concentration of 0.15 to 0.35 M. Compared with the separation of CEC_{wheat} on SP Sepharose® Fast Flow (cfr. Supra), a somewhat similar profile was obtained when screening with the *A. niger* enzyme, apart from a right hand shoulder, possibly indicating an additional HVXI inhibitor. In the profile resulting from screening with *B. subtilis* endoxylanase the right hand shoulder was very weak, indicating again that the method as used here allows to distinguish between inhibitors of different specificity. Figure 13 displays the chromatogram (–) of the final separation of HVXI on MonoS® with indication of the NaCl-gradient (-). With CEC on MonoS® the fractions containing inhibitor elute at NaCl concentration of 0.30 to 0.35 M.

Inhibitor partial molecular characterisation

Figure 14 shows the SDS-PAGE profile of HVXI with in lane 1 the low molecular mass markers (the size of the markers indicated on the left), in lane 2 pure inhibitor under non-reducing conditions and in lane 3 pure inhibitor under reducing conditions. Under non-reducing conditions, the purified inhibitor migrated as a double protein band with a molecular mass of ca. 40.0 kDa. In the presence of β-mercaptoethanol, the SDS-PAGE gel showed three protein bands with molecular masses of ca. 40.0 kDa; ca. 30.0 kDa and ca. 10.0 kDa. The pI of the inhibitor was at least ca. 9.3 and HVXI was not glycosylated.

The ca. 40.0 kDa and ca. 30.0 kDa polypeptides both share the same N-terminal amino acid sequence SEQ ID No. 3, indicating that the ca. 30.0 kDa polypeptide is proteolytically derived from the ca. 40.0 kDa protein. The N-

terminal amino acid sequence obtained for the ca. 10.0 kDa polypeptide is SEQ ID No. 6. The N-terminal amino acid sequences of the ca. 40.0 kDa and ca. 30.0 kDa polypeptide of HVXI show a high identity (94.4% identity in a 18-amino acid overlap and 90.0% identity in a 20-amino acid overlap respectively) with those of TAXI I and TAXI II. The N-terminal amino acid sequence of the ca. 10.0 kDa polypeptide of HVXI is less similar. It has 60.0% identity with the amino acid sequence of the ca. 10.0 kDa polypeptides of TAXI I and TAXI II in 15-amino acid overlaps.

Using a BLAST (version 2.0.10) search [26], the N-terminal sequence of the ca. 40.0 kDa and ca. 30.0 kDa polypeptides (amino acids 8-18) revealed a 72.7% identity in an 11-amino acid overlap with an internal sequence PITKDAHTSIY of a hypothetical protein from *Arabidopsis thaliana* (amino acids 344-354). The sequence of the ca. 10.0 kDa polypeptide showed 60.0% identity with the sequence GALATPGYPAAPYG of "osr40g3", a rice (*Oryza sativa* L.) protein.

Inhibition activities against xylanolytic enzymes

Figure 15 shows the activities of different levels of HVXI against five different endoxylanases, i.e. *A. aculeatus* (●), *A. niger* (■), *B. subtilis* (♦), *T. viride* (×) and rumen microorganism culture filtrate endoxylanases (▲).

HVXI has high activities against the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanases (family 11), low activity against the rumen micro-organism endoxylanases and little if any activity against the *A. aculeatus* endoxylanase (family 10). The maxima of inhibition are slightly above 95 % for the first two endoxylanases, ca. 92 % for the *B. subtilis* endoxylanase and ca. 15 % for the rumen micro-organism endoxylanases. It should be noticed that in the case of HVXI, the difference between the maxima of inhibition for the *A. niger* and the *T. viride* endoxylanases

on one hand and the maximum of inhibition for the *B. subtilis* endoxylanase on the other hand, is not as pronounced as in the case of TAXI I. Under the test conditions, different levels of HVXI (ca. 0.13, ca. 0.08 and ca. 0.27 µg respectively) reduce the activities of the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanase with 50 %.

Because after boiling (15 min, pH 5.0) no inhibition activity could be found against each of the mentioned endoxylanases, HVXI is heat sensitive.

Other AX hydrolysing enzymes, like an α-arabinofuranosidase and a β-xylosidase from *A. niger*, were not inhibited.

Discussion

We have purified and partially characterized HVXI, an endoxylanase inhibitor of barley. The SP Sepharose® Fast Flow profiles of CEC_{barley} however strongly suggest the presence of an additional endoxylanase inhibitor. HVXI is strongly related to TAXI I and TAXI II, since similar characteristics, N-terminal amino acid sequences and gel profiles under reducing and non-reducing conditions have been found for these wheat endoxylanase inhibitors (cfr. supra). In contrast, the inhibitor described by McLauchlan et al. [19] and Hessing and Happe [20] is a glycosylated, monomeric (single chained), basic protein with a molecular mass of 29 kDa. In addition, its N-terminal amino acid sequence showed 86% identity with the sequence of chitinase III from rice, whereas the N-termini of HVXI did not reveal such high identity with known proteins.

We believe that, much as TAXI I and TAXI II, HVXI occurs as two molecular forms, both with a molecular mass of ca. 40.0 kDa [16]. The first form exists as a single polypeptide chain. After proteolytic modification, it is transformed into the second form, which is composed of two disulfide linked subunits of ca. 30.0 kDa and ca. 10.0 kDa.

As in the case of TAXI I and TAXI II, we have strong indications that the non-proteolytically modified form is active, but to what extent the modified one is active, is still unclear. However, also here, it is reasonable to assume that the first form A is the precursor of the second form B and that, following proteolytic modification, the inhibitor becomes more or less active.

In the presence of HVXI, different endoxylanases were inhibited to a varying degree and HVXI showed a similar effect on the endoxylanase activities as TAXI I (cfr. supra). As in the case of TAXI I and TAXI II, part of the endoxylanase selectivity may be due to the fact that the enzymes tested belong to different families, i.e. family 10 or family 11. However, other factors should not be ignored, since, in the presence of the inhibitors, not all the family 11 enzymes tested reacted in the same way.

In general, proteinaceous enzyme inhibitors may be involved in plant defence mechanisms or in regulating certain metabolic activities in the plant [32, 9]. The endoxylanase inhibitor(s) in barley may have both functions. On the one hand, they may prevent the degradation of AX by phythopathogenic microorganisms, on the other hand they may regulate the AX degradation during germination. It has been shown that in extracts of germinating barley the xylan hydrolase activity appears several days later than (1-3),(1-4)-β-glucanases [33]. Although the endoxylanase genes are transcribed about 24 hours after those of the (1-3),(1-4)-β-glucanases [34], it has been suggested that the late appearance of the endoxylanase activity is due to a strong binding of these enzymes with the (aleurone) cell walls [33, 35]. However, the presence of endoxylanase inhibitors may also explain these observations.

Example 3: Isolation of endoxylanase inhibitors from commercial wheat flour, rye flour and barley whole meal using affinity chromatography with immobilised *B. subtilis* or *A. niger* endoxylanase

Experimental methods

Materials

N-hydroxysuccinimide(NHS)-activated Sepharose® 4 Fast Flow was purchased from Pharmacia Biotech (Uppsala, Sweden). Bakery enzyme preparation, Grindamyl® H 640 was from Danisco Cultor (Brabrand, Denmark). An *A. niger* endoxlanase preparation was obtained from Quest International (Naarden, the Netherlands). Rye (*Secale cereale* L., var. Halor) was from AVEVE (Landen, Belgium) and was milled with a Bühler MLU-202 mill. All other materials were as in examples 1 and 2.

Protein determination

Protein concentrations were determined in accordance with the Coomassie Brilliant Blue method of Bradford [21] with BSA as a standard.

Endoxylanase inhibition assay procedure

The inhibition activities were determined as described in the general experimental methods for examples 1 and 2.

Protein electrophoresis and sequencing

SDS-PAGE and protein sequencing were performed as described in the general experimental methods for examples 1 and 2.

Preparation of the affinity column

B. subtilis endoxylanase (XBS) was partially purified from a commercially available bakery enzyme preparation (Grindamyl® H 640) by elution on a cation exchange column (MonoS® HR 5/5) at pH 4.0 (25 mM sodium acetate) using a linear salt gradient from 0.0 to 1.0 M NaCl.

A. niger endoxylanase (XAN) was purified from a commercial Quest preparation by anion exchange chromatography (MonoQ® HR 5/5) at pH 8.0 (25 mM Tris/HCl) using a linear salt gradient from 0.0 to 1.0 M NaCl.

NHS-activated Sepharose® 4 Fast Flow (7.0 ml) was transferred to a small column of 15.0 ml, sealable at top and bottom. The matrix was washed with 1 mM HCl solution (70.0 ml). *B. subtilis* endoxylanase (50 mg) was dissolved in sodium bicarbonate buffer (0.2 M, pH 8.3; 7.0 ml) containing 0.5 M NaCl. Just before coupling, the activated matrix was washed with the same sodium bicarbonate buffer. The XBS solution (7.0 ml) was applied on top of the matrix. When ca. 4.0 ml of the enzyme solution had entered the matrix, the column was sealed and the coupling reaction was started. Coupling was performed at room temperature for 2.5 h, while the mixture was shaken. After the incubation period, the uncoupled endoxylanase was removed by washing with an ethanol amine solution (0.5 M, pH 8.3) containing 0.5 M NaCl (35.0 ml). An additional 5.0 ml of the same ethanol amine solution was added, the column was sealed and the mixture was shaken and allowed to react for 4 h at room temperature. The ethanol amine solution was replaced once during this incubation period. Finally, the matrix was washed successively with glycine solution (0.1 M, pH 3.0) containing 0.5 M NaCl (35.0 ml) and ethanol amine solution (0.5 M, pH 8.3) containing 0.5 M NaCl (35.0 ml).

The preparation of the affinity column with immobilised *A. niger* endoxylanase used an identical

procedure, but starting from a XAN solution (50 mg in 7.0 ml).

Purification method using the XBS affinity column

Step I. Extraction and concentration

The extraction of wheat flour and barley whole meal, the successive concentration and partial purification were performed as described in the general experimental methods for examples 1 and 2 resulting in CEC_{wheat} and CEC_{barley} material. From 2.5 kg of wheat flour, approximately 4.4 g of CEC_{wheat} material was obtained.

Rye flour (2.0 kg) was extracted with 20.0 l of 0.1% (w/v) ascorbic acid in water. The extract was further treated as described for wheat and barley in the general experimental methods for examples 1 and 2 yielding the CEC_{rye} material.

Step II. Purification by affinity chromatography (AFC)

Batches of the CEC_{wheat} (400 mg), CEC_{rye} (250 mg) and CEC_{barley} (250 mg) material were dissolved in sodium acetate buffer (25 mM, pH 5.0; 25 ml, 10 ml and 10 ml respectively) containing 0.2 M NaCl and applied to the affinity column with immobilised *B. subtilis* endoxylanase (equilibrated with the same buffer) at a flow rate of 0.33 ml/min. Proteins with endoxylanase inhibiting activity were eluted with 5.0 ml of a 0.25 M Tris/HCl buffer (pH 10.0) at a flow rate of 1.0 ml/min.

The eluted fractions were neutralized immediately with acetic acid (1.0M) and dialysed against sodium acetate buffer (25 mM, pH 4.0, 48 h) or were subjected to a buffer exchange (same buffer) using a PD-10 column, resulting in the AFC_{wheat} (30.5 mg protein in 187 ml), AFC_{rye} and AFC_{barley} material, respectively.

Step IIIa. Purification of wheat endoxylanase inhibitors by CEC

Three separate batches of the AFC_{wheat} solution (62 ml) was applied on a MonoS® HR 5/5 column, previously equilibrated with sodium acetate buffer (25 mM, pH 4.0). The bound proteins were eluted with a linear gradient of 0.0-0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min. This resulted in two inhibitor protein fractions, one eluting at 0.22-0.30 M NaCl (wheat inhibitor fraction I) and one eluting at 0.36-0.44 M NaCl (wheat inhibitor fraction II). The collected inhibitor fractions I (13.8 mg protein in 36.0 ml) and II (5.5 mg protein in 33.0 ml) were dialysed against sodium acetate buffer (25 mM, pH 5.0) and sodium phosphate buffer (20 mM, pH 6.5) respectively.

In a final step wheat inhibitor fractions I and II were separated on MonoS® HR 5/5 columns, equilibrated with sodium acetate buffer (25 mM, pH 5.0) and sodium phosphate buffer (20 mM, pH 6.5) respectively. In both cases, elution was with a linear gradient of 0.0-0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min.

Step IIIb. Purification of rye endoxylanase inhibitors by CEC

The AFC_{rye} material was further fractionated on a MonoS® column, equilibrated with a 25 mM sodium acetate buffer (pH 4.0). The bound proteins were eluted with a linear gradient of 0.0 to 0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min and collected in 0.5 ml fractions. This resulted in four separate inhibitor solutions, which were subjected to a buffer exchange [sodium acetate buffer (25 mM, pH 5.0)] (=rye inhibitor fractions I-IV).

The different rye inhibitor fractions were seperated with the MonoS® column, equilibrated with a 25 mM sodium acetate buffer (pH 5.0) and using the same elution conditions as described above. Fractions (0.5 ml) were collected and assayed for their ability to inhibit the *A. niger*, *B. subtilis* and *T. viride* endoxylanases.

Purification method using the XAN affinity column

The CEC_{wheat} material(400 mg) was dissolved in sodium acetate buffer (25 mM, pH 5.0; 25 ml) 0.2 M NaCl and applied to the affinity column with immobilised *A. niger* endoxylanase (equilibrated with the same buffer) at a flow rate of 0.33 ml/min. A protein fraction containing endoxylanase inhibiting activity was eluted with 5.0 ml of deionised water. More endoxylanase inhibiting proteins were eluted with 5.0 ml of a 0.25 M Tris/HCl buffer (pH 10.0). The flow rate during elution was 1.0 ml/min.

Results

Purification by affinity chromatography with immobilised XBS

Figure 16 shows the SDS-PAGE profiles of wheat inhibitor fractions I and II under reducing conditions (lanes 2 and 1 respectively) and non-reducing conditions (lanes 4 and 3 respectively), of the AFC_{wheat} material, the CEC_{wheat} material and the low molecular mass markers (the size of the markers indicated on the right) under non-reducing conditions (lanes 5, 6 and 7 respectively).

SDS-PAGE analysis under reducing and non-reducing conditions of the AFC_{wheat}, AFC_{rye}, AFC_{barley} and of the wheat and rye inhibitor fractions obtained after fractionation on MonoS® at pH 4.0, showed that all isolated proteins are of the same general molecular structure as described for TAXI I and TAXI II in examples 1 and 2, i.e. a form A, which consists of a single polypeptide chain of ca. 40 kDa, and a form B, which consists of two disulfide linked subunits of ca. 30 and ca. 10 kDa. Hence, the *B. subtilis* enzyme has a high selective binding affinity for the 'TAXI'-like proteins, present in wheat, rye and barley.

Purification of wheat endoxylanase inhibitors

Figure 17 shows the chromatogram (—) and the NaCl gradient (—) of the separation of AFC_{wheat} solution on a MonoS® column at pH 4.0, resulting in inhibitor fractions I and II.

The eluate (10 µl) of the affinity column contained high inhibition activity against the *A. niger* (95.2% inhibition) and the *B. subtilis* (87.3% inhibition) endoxylanases. Wheat inhibitor fraction I (10 µl) also inhibited both enzymes to a great extent (92.1% and 84.3% inhibition respectively) whereas wheat inhibitor fraction II (20 µl) inhibited the *A. niger* endoxylanase (13.4% inhibition) much less than the *B. subtilis* endoxylanase (81.4% inhibition). Fractionation of inhibitor fraction I on a MonoS® column at pH 5.0 and similar fractionation of inhibitor fraction II at pH 6.5 resulted in two and three distinct inhibitor peaks respectively.

Figure 18 shows the chromatogram (—) and the NaCl gradient (—) of the separation of the inhibitor fraction I on a MonoS® column at pH 5.0.

Figure 19 shows the chromatogram (—) and the NaCl gradient (—) of the separation of the inhibitor fraction II on a MonoS® column at pH 6.5.

The two peaks resulting from inhibitor fraction I were very close together and difficult to separate on MonoS®. At pH values above 5.0, these inhibitors barely bound on the column. They had high inhibition activities against the *A. niger* and the *B. subtilis* endoxylanases. The first two peaks resulting from inhibitor fraction II had pronounced activities against both endoxylanases whereas the third one inhibited the *B. subtilis* endoxylanase to a much higher extent

than the *A. niger* endoxylanase. The activity against the *A. niger* enzyme probably at least partially, originated from the second peak that had a small overlap with the third one.
These findings suggest that, in commercial wheat flour, up to five, or even more, inhibitors occur. Based on their elution behaviour on MonoS® and their inhibition activities against the *A. niger* and the *B. subtilis* endoxylanases, the inhibitors from inhibitor fraction I and the inhibitor(s) corresponding to the third peak obtained by MonoS® at pH 6.5 of inhibitor fraction II, may correspond to what is considered TAXI I and TAXI II respectively, as described above for var. Soissons (Example 1).

Purification of rye endoxylanase inhibitors

Figure 20 shows the chromatogram (—) and the NaCl gradient (—) of the separation of the AFC_{rye} material on a MonoS® column at pH 4.0. This chromatogram, combined with inhibition activity measurements and SDS-PAGE analysis of the collected fractions (gels not shown), suggested the presence of several 'TAXI'-like endoxylanase inhibitors in rye (SCXI or *Secale cereale* L. xylanase inhibitor). Four rye inhibitor fractions I-IV, as indicated in figure 20, were discerned.

Figure 21 shows the NaCl gradient (—) and the chromatograms of the separations of the rye inhibitor fractions I (—), II (— —), III (- -) and IV (—) on MonoS® at pH 5.0. After fractionation of the rye inhibi-tor fractions I-IV at least five different inhibitor peaks SCXI I-V could be distinguished, as indicated in figure 21.

When analysed with SDS-PAGE, all the inhibitors were structurally similar to TAXI and HVXI, as described in examples 1 and 2 (gel not shown). Furthermore, SCXI IV and V were electrophoretically pure, while SCXI I, II and III still contained some minor impurities. Because of the small differences in elution volume of these inhibitors and the

similar SDS-PAGE profiles, it's possible that some of these five distinct inhibitor peaks (in particular SCXI II and III) contained more than one 'TAXI'-like inhibitor.

The different rye inhibitors, in particular SCXI IV and V, reduced the activity of the endoxylanases of *A. niger*, *B. subtilis* and *T. viride* to the same extent, indicating that these inhibitors have similar specificities. In contrast, the activities of TAXI I and II from wheat against the *A. niger* endoxylanase are clearly different, the former being a strong inhibitor of this enzyme and the latter having little if any effect on its activity. Moreover, the levels of SCXI IV and V needed to reduce the activity of the endoxylanases tested by 50%, were comparable to those needed if TAXI I and HVXI are to yield the same effect.

We can conclude that rye contains a family of endoxylanase inhibitors (with at least five members) with similar structures and specificities. These characteristics corresponds well with the properties of TAXI (I) and HVXI as described in example 1 and 2. Therefore, SCXI I-V are their rye homologues.

Some preliminary work using this approach comprising affinity chromatography with immobilised *B. subtilis* endoxylanase to purify endoxylanase inhibitors from durum wheat has shown promising results with 'TAXI'-like inhibitors binding selectively to the affinity column.

Purification by affinity chromatography with immobilised XAN

Using the affinity column with immobilised *A. niger* endoxylanase and different elution conditions, we obtained two separate inhibitor fractions. The material eluted with deionised water, contained mainly proteins of about 30 kDa (SDS-PAGE), while the protein fraction eluted with the

Tris/HCl buffer, consisted of 'TAXI'-like inhibitors. Determination of the N-terminal amino acid sequence of the 30 kDa proteins yielded sequences identical to those reported by Hessing and Happe [20] and McLauchlan et al [19]. Hence, the *A. niger* enzyme has a high selective binding affinity for the 'TAXI'-like proteins as well as for the non-'TAXI' inhibitors, present in wheat. This demonstrates that the purification of endoxylanase inhibitors using affinity chromatography with an immobilised endoxylanase is not limited to the 'TAXI'-like inhibitors.

A similar approach using affinity chromatography with immobilised endoxylanase inhibitors is expected, based on the results presented above, to be a very powerful tool for the purification of various endoxylanases (example 4).

Example 4: Isolation of an *A. niger*, var. *awamori* endoxylanase from a commercial enzyme preparation using affinity chromatography with immobilised 'TAXI'-like endoxylanase inhibitors

Experimental methods

Materials

Enzyme preparation containing endoxylanase of *A. niger*, var. *awamori* was obtained from Quest international (Naarden, Netherlands). All other materials were as described in example 1, 2 and 3.

Endoxylanase activity assay procedure

The endoxylanase activities were determined with the Xylazyme-AX method. Appropriately diluted sample (1.0 ml), containing endoxylanase and prepared in sodium acetate buffer (25 mM, pH 5.0) was incubated for 60 min at 40 °C with an AZCL-AX substrate tablet. The reaction was terminated with a 1.0% (w/w) Tris solution. The remainder of the procedure was similar to that for endoxylanase inhibition activity determination.

Protein electrophoresis

SDS-PAGE was performed as described in the general experimental methods for examples 1 and 2.

Preparation of the affinity column and endoxylanase purification method

The mixture of 'TAXI'-like endoxylanase inhibitors (42.0 mg) obtained after the affinity purification step, as described in example 3, were immobilised on the same carrier using a similar procedure as for the *B. subtilis* endoxylanase, as also explained in example 3.

The *A. niger*, var. *awamori* enzyme preparation (20.0 mg) was extracted for 30 min at room temperature with sodium acetate buffer (25 mM, pH 5.0; 5.0 ml) containing NaCl (0.2 M) and the resulting suspension was centrifuged (10000 *g*, 30 min, 7 °C). The supernatant was loaded on the affinity column with the immobilised endoxylanase inhibitors, equilibrated previously with the same NaCl containing acetate buffer. The proteins retained on the column were eluted with Tris buffer (250 mM, pH 8.0; 5.0 ml) and immediately neutralised with acetic acid solution(1.0 M).

Results

Most endoxylanase activity (ca. 94%) of the enzyme preparation was retained on the affinity column, presumably by interacting with the 'TAXI'-like endoxylanase inhibitors covalently linked to the matrix. After elution most of the endoxylanase activity (ca. 84%) could be recovered. The eluate comprised mainly of the endoxylanase, which has a relative molecular mass of ca. 23 kDa. Only three additional bands with lower molecular masses and of very low intensity could be observed.

Figure 21 shows the SDS-PAGE profiles of the fraction eluted from the affinity column (lane 1), the starting material (lane 2) and low molecular mass markers (lane 3, with the size of the markers indicated on the right).

Example 5. Wheat protein encoding DNA sequences

A BLAST search (TBLASTN 2.1.2, www.ncbi.nlm.nih.gov) in the database with non-human and non-mouse EST sequences using SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 5 (this patent application) in combination with SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 and SEQ ID No. 19 as described in the patent application by Sibbesen and Sørensen [36] produced significant alignments with 5 cDNA clones of wheat:

IDENTIFIERS dbEST Id: 5493910 EST name: WWS020.H4R000101 GenBank Acc: BE420158

IDENTIFIERS dbEST Id: 5493479 EST name: WWS016.G1R000101 GenBank Acc: BE419727

IDENTIFIERS dbEST Id: 5504159 EST name: SUN002.E06R991208 GenBank Acc: BE430407

IDENTIFIERS dbEST Id: 5452003 EST name: CSB006D03F990908 GenBank Acc: BE402285

IDENTIFIERS dbEST Id: 6889613 EST name: WHE1409_B12_C23ZS GenBank Acc: BF428535

The cDNA clones were aligned with the SEQUENCHER™ programme and using SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 5 (this patent application) in combination with SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 and SEQ ID No. 19 as described in the patent application by Sibbesen and Sørensen [36] , the amino acid sequence of TAXI I was obtained within one reading frame. The present invention thus features a TAXI I variant having the amino acid sequence shown in SEQ ID No. 7:

LPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAY
PAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAXSLXHTXFVANTTDGXKPVSKVNVGVLA
ACAPSKLLASLPRGSTGVAGLADSGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGPL

PWPQFTQSMPYTPLVTKGGSPAHYISARSIEVGDTRVPVSEGALATGGVMLSTRLPYVLLR
RDVYRPLVDAFTKALAAQHANGAPVARAVEPVAPFGVCYDTKTLGNNLGGYAVPNVQLGLD
GGSDWTMTGKNSMVDVKXGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKR
LGFSRLPHFTGCGGL.

The present invention thus also features a TAXI I variant having the amino acid sequence shown in Seq. ID no. 8:
LPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAY
PAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAXSLXHTXFVANTTDGXKPVSKVNVGVLA
ACAPSKLLASLPRGSTGVAGLANSGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGPV
PWPQFTQSMPYTPLVTKGGSPAHYISARFIEVGDTRVPVSEGALATGGVMLSTRLPYVLLR
RDVYRPLVDAFTKALAAQHANGAPVARAVEAVAPFGVLYDTKTLGNNLGGYAVPNVQLGLD
GGSDWTMTGKNSMVDVKXGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKR
LGFSRLPHFTGCGGL.

Or the present invention features microheterogenic TAXI I variants having the amino acid sequence shown in Seq. ID no. 9:
LPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAY
PAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAXSLXHTXFVANTTDGXKPVSKVNVGVLA
ACAPSKLLASLPRGSTGVAGLAXSGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGPX
PWPQFTQSMPYTPLVTKGGSPAHYISARXIEVGDTRVPVSEGALATGGVMLSTRLPYVLLR
RDVYRPLVDAFTKALAAQHANGAPVARAVEXVAPFGVXYDTKTLGNNLGGYAVPNVQLGLD
GGSDWTMTGKNSMVDVKXGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKR
LGFSRLPHFTGCGGL, wherein X consists of an amino acid of the group D,N,V,L,S,F,P,A and C.

Using a BLAST search (TBLASTN 2.1.2), the overall protein sequence of TAXI I shows significant homology with carrot mRNA encoding for an extracellular dermal glycoprotein (EDGP) (44%), an *Arabidopsis thaliana* putative extracellular dermal glycoprotein precursor (F15K9.16) mRNA (41%), a soybean Bg gene for a basic 7S globulin (41%) and with a *Cicer arietinum* mRNA for a putative extracellular glycoprotein (ORF1) (41%).

On the basis of the obtained nucleotide sequences of TAXI I, two other homologous cDNA clones from rice (GenBank Number: AU068900 and AU068987) were obtained from which we could determine some more upstream lying nucleotides.

From the above, the following consensus sequence of TAXI I (SEQ ID No. 10) (including ca. 95% of the coding sequence and the poly-adenylation site) was obtained:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCTCTCGTCTGGTCCACGTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCTCCCAGCTGCGGC
AGCGATAAGCACGACAAACCGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCG
CCGCMKGGAGCCTCTYCCACACGARRTTCGTGGCCAACACCACCGACGGGARYAARCCGGT
GAGCAAGGTGAACGTCGGGGTCCTGGCGGCGTGCGCGCCGAGCAAGCTCCTGGCGTCGCTG
CCCCGGGGCTCCACGGGCGTGGCCGGGCTCGCGGACTCCGGCCTGGCGCTGCCGGCGCAGG
TGGCGTCCGCGCAGAAGGTCGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCCTGG
CGTGGCCATCTTCGGCGGCGGCCCGCTCCCGTGGCCGCAATTCACGCAGTCGATGCCCTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCCGCCAGGTCCATCGAAG
TGGGGGACACCCGCGTCCCCGTATCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTGCCCTACGTCTTGCTCCGCCGCGACGTGTACCGCCCGTTGGTGGACGCGTTC
ACCAAGGCCCTGGCGGCGCAGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGCCTG
TGGCGCCGTTCGGGGTGTGCTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGC
GGTGCCCAACGTCCAGCTGGGGCTCGATGGCGGSAGTGACTGGACGATGACCGGGAAGAAC
TCGATGGTGGACGTCAAGCMRGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGG
CGGCCGGCGACGGCAGGGCGCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGT
GCTCGACTTCGACATGGAGAAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGT
TGCGGCGGCCTGTAATAATAAATCTGTTTAACGACAGGTGGATTCGTCCACTACTGCGTGT
AATAAATAAGGGAAGAAACACTTTCCATCAGTGGTTTCAT.

As a next step, based on the consensus TAXI I sequence (SEQ ID No. 10) 4 primers were designed. Primer 1 is GCCACCTCCCTCTACACAATC (SEQ ID No. 31). Primer 2 is GTAGTGGACGAATCCACCTGTC (SEQ ID No. 32). Primer 3 is CGCAATTCACGCAGTCGATG (SEQ ID No. 33). Primer 4 is

CCCAGCGTCTTCGTGTCGTAG (SEQ ID No. 34). Primers 1 and 2 are positioned at the flanks of the sequence, while primers 3 and 4 are internal primers. Primers were ordered from Genset (Paris, France).

PCR reactions were performed in 35 µl using 0.05 Units HotStarTaq DNA Polymerase (Qiagen, Hilden ,Germany), commercially supplied buffer (Qiagen), 200 µM of each dNTP, 1 µM of each primer and 50 ng total genomic DNA as template, prepared as described [37]. The reaction mixtures were subjected to incubation for 15 min at 95°C, followed by 30 cycles of 1 min at 94°C, 90 s at 57°C, 2 min at 72°C and a final incubation for 15 min at 72°C on a UNO II thermocycler (Biometra, Göttingen, Germany).

PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, California, USA). The ligation mixtures, composed of 1 µl PCR 4-TOPO vector, 1 µl salt solution (supplied in the kit) and 4 µl fresh PCR product, were incubated for 5 min at 20 °C. Two µl of the ligation mixture was added to a vial of TOP10 One Shot Chemically Competent E. coli (supplied in the kit) and incubated on ice for 10 min. Subsequently, the reactions were incubated at 42°C for 30 seconds and transferred to ice. After addition of 250 µl SOC medium (supplied in the kit) and incubation in a shaker at 37°C for 1 hour, the transformations were spread on selective (ampicillin) agar plates and incubated overnight at 37°C.

DNA sequencing of the cloned PCR products was done using QIAprep Spin Miniprep (Qiagen) purified plasmids, vector specific primer and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Sequencing gels were run on a 377 ABI PRISM DNA Sequencer (Applied Biosystems).

Results

Clones representing partial xylanase inhibitor sequences were obtained from PCR-amplified genomic DNA from bread wheat (*Triticum aestivum* cultivar Soissons and cultivar Estica), durum wheat (*Triticum durum* cultivar Mexicali) and the diploid wild wheat *Aegilops tauschii*. The xylanase inhibitor type I or II was identified by alignment of the encoded amino acid sequences with peptide sequences identified from native TAXI type I and II. Besides sequences related to type I and type II, a divergent sequence (called type III) was identified. From alignments with cDNA sequences it is clear that none of the cloned fragments contained introns.

Five clones are presented. The first three sequences represent type I inhibitors. The fourth sequence represents a type II inhibitor. The last sequence represents a type III inhibitor. The sequences of five cloned PCR products are as follows:

SEQ ID No. 15 is part of a xylanase inhibitor gene termed TAXI-I.01 from *Triticum aestivum* cultivar Soissons:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCTCTCGTCTGGTCCACGTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCGCCCAGCTGCGGC
AGCAACAGGCACAACAAGCCGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCG
CCGCAGGGAGCCTCTCCCACACGAGATTCGTGGCCAACACCACCGACGGGAGCAAGCCGGT
GAGCAAGGTGAACGTCGGGGTCCTGGCGGCGTGCGCGCCGAGCAAGCTCCTGGCGTCGCTG
CCCCGGGGCTCCACGGGCGTGGCCGGGCTCGCGAACTCCGGCTTGGCGCTGCCGGCGCAGG
TGGCATCCGCGCAGAAGGTCGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCTTGG
CGTGGCCATATTTGGCGGCGGCCCGGTCCCGTGGCCGCAATTCACGCAGTCGATGCCTTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCGGCCAGGTCCATTGTAG
TGGGGGACACCCGCGTCCCCGTATCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTACCCTACGTCTTGCTCCGCCCCGACGTGTACCGCCCGTTGATGGACGCGTTT
ACCAAGGCCCTGGCGGCGCAGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGGCTG
TGGCGCCGTTCGGGGTGTGCTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGC
GGTGCCCAACGTCCAGCTGGGGCTCGATGGCGGCAGTGACTGGACGATGACCGGGAAGAAC

TCGATGGTGGACGTCAAGCAAGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGG
CGGCCGGCGACGGCAGGGCGCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGT
GCTCGACTTCGACATGGAGAAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGT
TGCGGCGGCCTGTAATAATAAATCTGTTTAACGACAGGTGGATTCGTCCACTAC.

SEQ ID No. 16 is part of a xylanase inhibitor gene termed TAXI-I.02 from *Triticum aestivum* cultivar Estica:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCTCTCGTCTGGTCCACGTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCTCCCAGCTGCGGC
AGCGATAAGCACGACAAACCGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCG
CCGCAGGGAGCCTCTCCCACACGAGATTCGTGGCCAACACCACCGACGGGAGCAAGCCGGT
GAGCAAGGTGAACGTCGGGGTCCTGGCGGCGTGCGCGCCGAGCAAGCTCCTGGCGTCGCTG
CCCCGGGGCTCCACGGGCGTGGCCGGGCTCGCGAACTCCGGCTTGGCGCTGCCGGCGCAGG
TGGCATCCGCGCAGAAGGTCGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCCTGG
CGTGGCCATATTTGGCGGCGGCCCGGTCCCGTGGCCGCAATTCACGCAGTCGATGCCTTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCGGCCAGGTCCATTGTAG
TGGGGGACACCCGCGTCCCCGTACCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTACCCTACGTCTTGCTCCGCCCCGACGTGTACCGCCCGTTGATGGACGCGTTC
ACCAAGGCCCTGGCGGCGCAGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGGCTG
TGGCGCCGTTCGGGGTGTGCTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGC
GGTGCCCAACGTCCAGCTGGGGCTCGATGGCGGCAGTGACTGGACGATGACCGGGAAGAAC
TCGATGGTGGACGTCAAGCAAGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGG
CGGCCGGCGACGGCAGGGCGCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGT
GCTCGACTTCGACATGGAGAAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGT
TGCGGCGGCCTGTAATAATAAATCTGTTTAACGACAGGTGGATTCGTCCACTAC.

SEQ ID No. 17 is part of a xylanase inhibitor gene termed TDXI-I.01 from *Triticum durum* cultivar Mexicali:

CGCAATTCACGCAGTCGATGCCTTACACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCA
CTACATCTCGGCCAGGTCCATTGTAGTGGGGGACACCCGCGTCCCCGCCGTATCGGAGGGC
GCGCTCGCCACCGGCGGCGTGATGCTCAGCACGAGGCTACCCTACGTCTTGCTCCGCCCCG
ACGTGTACCGCCCGTTGATGGACGCGTTCACCAAGGCCCTGGCGGCGCAGCATGCCAACGG
AGCGCCCGTGGCGCGCGCAGTGGAGGCTGTGGCGCCGTTCGGGGTGTGCTACGACACGAAG
ACGCTGGGCAACAACCTCGGCGGGTACGCGGTGCCCAACGTCCAGCTGGGGCTCGATGGCG

GCAGTGACTGGACGATGACCGGGAAGAACTCGATGGTGGACGTCAAGCAAGGGACGGCGTG
CGTTGCGTTCGTGGAGATGAAGGGAGTGGCGGCCGGCGACGGCAGGGCGCCGGCGGTGATC
CTCGGAGGGGCCCAGATGGAGGACTTCGTGCTCGACTTCGACATGGAGAAGAAGCGGCTCG
GGTTTAGCAGGCTGCCGCACTTTACGGGTTGCGGCGGCCTGTAATAATAAATCTGTTTAAC
GACAGGTGGATTCGTCCACTAC.

SEQ ID No. 18 is part of a xylanase inhibitor gene termed ATXI-II.01 from *Aegilops tauschii* variety Acc2220051:

GCCACCTCCCTCTACACAATCCCCTTCCACCAGGGCGCCAGCCTCGTCCTTGACATCGCCG
GCCCGCTCGTCTGGTCCACGTGCCAGCGCGGCGATCTGCCGACAGATATCCCGTGCAGTAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCGCCCAGCTGCGGC
AGCGGCAGCCACGACAAGCAATGCACGACGTACCCATCCAACCCGGTCACCGGCGCGTGCG
CCGCCGGGAGCCTCGCCCGCACGACGCTCATAGCCGACACCACCGACGGGAATAACCCGGT
GAGCCAGGTGTACGTCCGGATCCTGGCGGCGTGCGCGCCGAGAAAGCTCCTGGCGTCGCTG
CCCCGCGGCTCCATGGGCGTCGCCGGGCTAGGGGGCTCCGGCCTGGCGCTGCCGGCGCAGG
TGGCGTCCACCCAGAAGGTCGCCAACAAGTTTCTCCTCTGCCTCCCCAGCGGCGGCCCTGG
CGTGGCCATCTTCGGCGGCGGCCCGCTCCCGTGGCCGCAATTGACGCAGTCGATGCCGTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCCGTCAAGGCCATCCAAC
TGGAGGACACCCGCGTCTCCGTCTCAGAGCGCGTGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTGCCCTACGCCTTGCTCCGCCACGACGTCTACCGCCCGTTGGTGGACGCGTTC
ACCAAGGCCCTGGCGGCGCAGCCTGCCAACGGAGCGCCCGTGGCGCGCGCCGTGAAGCCTG
TGGCACCGTTCGAGCTGTGCTACGACACGAAGACGCTGGG.

SEQ ID No. 40 is part of a xylanase inhibitor gene termed TAXI-III from *Triticum aestivum* cultivar Soissons:

GCCACCTCCCTCTACACAATCCCATTCCACTACGGCGCCAACATCGTGGTCGACACCGCCG
GACCGCTCGTCTGGTCCACGTGCGCACCCGACCACCTGCCGGCGGCGTTCCCGTGCAAGAG
CGCCACCTGCAGGCTCGCGAACAAGTACCACGTCCCGAGCTGCAGCGAGAGCGCGGCTGAC
AAGCTCTGCGACCACAGTCACAAGGTGTGCAGGGCCTTCCCGTACAACCCGGTCACCGGCG
CGTGCGCGGCCGGGGACCTGATCCACACCAGGTTCGTCGCCAACACCACCGACGGAAAAAA
CCCGGTGAGCCAGGTGAACGTTCGGGCCGTGGCGGCGTGCGCGCCAAGCAAACTCCTCGAG
TCGCTGCCGCAGGGCGCCTCGGGCGTGGCGGGGCTCGCGGGCTCCGACCTGGCGCTGCCGG
CGCAGGTGGCGTCCGAGCAGAAGGTCTCCAACAAGTTCCTCCTCTGCCTCCCTCGCGGCCT
CTCAAGCGACCCCGGCGTGGCCGTCTTCGGCGGCGGCCCGCTCCACTTCATGGCGCGGCCG
GAGAGGGACTACACGAAGGAGCTGGCCTACACGCCGCTCGTCGCCAAGAAGGGCAACCCCG

CGCACTACATCTCGATCAAGTCCATCGCCGTGGAGAGCGCCCGCGTGCCCGTCCCGGCGCA
GGCGCTCGCCACCGGTGGGGCGGTGCTCTGCACGAGGTCGCCCTTCACCCTGCTCCGCTCC
GACGTGTTCCTCCCGTTGGTGGACGCGTTCACCAAGGCCCTGGCGAAGCAGGGTGCGCAGG
GCGGGCCCGTGGCGAAAGCGGTGAAGCCCTACGCGCCGTTCCAGCTGTGCTACGACACGAA
GACGCTGGG.

The encoded xylanase inhibitor amino acid sequences are as follows:

SEQ ID No. 19 is part of a xylanase inhibitor type I encoded by SEQ ID No. 15:

ATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAYPAPGCPAPSCG
SNRHNKPCTAYPYNPVSGACAAGSLSHTRFVANTTDGSKPVSKVNVGVLAACAPSKLLASL
PRGSTGVAGLANSGLALPAQVASAQKVANRFLLCLPTGGLGVAIFGGGPVPWPQFTQSMPY
TPLVTKGGSPAHYISARSIVVGDTRVPVSEGALATGGVMLSTRLPYVLLRPDVYRPLMDAF
TKALAAQHANGAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNVQLGLDGGSDWTMTGKN
SMVDVKQGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFSRLPHFTG
CGGL.

SEQ ID No. 20 is part of a xylanase inhibitor type I encoded by SEQ ID No. 16:

ATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAYPAPGCPAPSCG
SDKHDKPCTAYPYNPVSGACAAGSLSHTRFVANTTDGSKPVSKVNVGVLAACAPSKLLASL
PRGSTGVAGLANSGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGPVPWPQFTQSMPY
TPLVTKGGSPAHYISARSIVVGDTRVPVPEGALATGGVMLSTRLPYVLLRPDVYRPLMDAF
TKALAAQHANGAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNVQLGLDGGSDWTMTGKN
SMVDVKQGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFSRLPHFTG
CGGL.

SEQ ID No. 21 is part of a xylanase inhibitor type I encoded by SEQ ID No. 17:

QFTQSMPYTPLVTKGGSPAHYISARSIVVGDTRVPAVSEGALATGGVMLSTRLPYVLLRPD
VYRPLMDAFTKALAAQHANGAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNVQLGLDGG
SDWTMTGKNSMVDVKQGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKRLG
FSRLPHFTGCGGL.

SEQ ID No. 22 is part of a xylanase inhibitor type II encoded by SEQ ID No. 18:

ATSFYTIPFHQGASLVLDIAGPLVWSTCQRGDLPTDIPCSSPTCLLANAYPAPGCPAPSCG
SGSHDKQCTTYPSNPVTGACAAGSLARTTLIADTTDGNNPVSQVYVRILAACAPRKLLASL
PRGSMGVAGLGGSGLALPAQVASTQKVANKFLLCLPSGGPGVAIFGGGPLPWPQLTQSMPY
TPLVTKGGSPAHYISVKAIQLEDTRVSVSERVLATGGVMLSTRLPYALLRHDVYRPLVDAF
TKALAAQPANGAPVARAVKPVAPFELCYDTKTL.

SEQ ID No. 41 is part of a xylanase inhibitor type III encoded by SEQ ID No. 40:

ATSLYTIPFHYGANIVVDTAGPLVWSTCAPDHLPAAFPCKSATCRLANKYHVPSCSESAAD
KLCDHSHKVCRAFPYNPVTGACAAGDLIHTRFVANTTDGKNPVSQVNVRAVAACAPSKLLE
SLPQGASGVAGLAGSDLALPAQVASEQKVSNKFLLCLPRGLSSDPGVAVFGGGPLHFMARP
ERDYTKELAYTPLVAKKGNPAHYISIKSIAVESARVPVPAQALATGGAVLCTRSPFTLLRS
DVFLPLVDAFTKALAKQGAQGGPVAKAVKPYAPFQLCYDTKTL.

Example 6. Rye protein encoding DNA sequences.

PCR was also performed on genomic DNA from rye (*Secale cereale* cultivar Halo) using primers 3 (SEQ ID No. 33) and 4 (SEQ ID No. 34) according to the procedures as descibed above. Two clones are presented. The sequences of two cloned PCR products are as follows:

SEQ ID No. 23 is an internal fragment of a xylanase inhibitor gene termed SCXI-01 from *Secale cereale* cultivar Halo:

CGCAATTCACGCAGTCGATGCAGTACACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCA
CTACATCTCGCTGAAGTCCATCAAAGTGGACAACACCGGCGTCACCGTCTCGCAGAGCGCG
TTCGCCACCGGCGGCGTGATGCTGAGCACGAGGCTGCCCTACGCCCTGCTCCGCCGCGACG
TGTACCGCCCGTTGGTGGACGCGTTCACCAAGGCCCTGGCGGCGCAGCCTGCCAACGGAGC
GCCCGTGGCGCGCGCAGTGCAGCCCGTGGCGCCGTTCGGGGTGTGCTACGACACGAAGACG
CTGGG

SEQ ID No. 24 is an internal fragment of a xylanase inhibitor gene termed SCXI-02 from Secale cereale cultivar Halo:

CGCAATTCACGCAGTCGATGCAGTACACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCA
CTACATCTCGCTCAAGTCCATCAAAGTGGACAACACCGGCGTCACCCTCTCGCAGAGCGCG
CTCGCCACCGGCGGCGTGATGCTCAGCACGAGGCTGCCCTACGCCCTGCTCCGCAGCGACG
TGTACCGCCCGTTGGTGGACGCGTTCACCAAGGCCCTGGCGGCGCAGCCTGTCAACGGAGC
GCCCGTGGCGCGCGCGGTGAAGCCCGTGGAGCCGTTCGGGGTGTGCTACGACACGAAGACG
CTGGG

The encoded xylanase inhibitor amino acid sequences are as follows:

SEQ ID No. 25 is part of a xylanase inhibitor encoded by SEQ ID No. 23:

QFTQSMQYTPLVTKGGSPAHYISLKSIKVDNTGVTVSQSAFATGGVMLSTRLPYALLRRDV
YRPLVDAFTKALAAQPANGAPVARAVQPVAPFGVCYDTKTL.

SEQ ID No. 26 is part of a xylanase inhibitor encoded by SEQ ID No. 24:

QFTQSMQYTPLVTKGGSPAHYISLKSIKVDNTGVTLSQSALATGGVMLSTRLPYALLRSDV
YRPLVDAFTKALAAQPVNGAPVARAVKPVEPFGVCYDTKTL.

Example 7. Rice protein encoding DNA sequences.

A FASTA3 search in the EMBL ESTs library using SEQ ID No. 15 (this patent application) produced significant alignment with two non-overlapping cDNA clones of rice with accession numbers D15808 and C26221. The cDNA sequence D15808 shows an overlap with the 5' part (152 nt) of SEQ ID No. 15 (this patent application). The cDNA sequence C26221 overlaps internally with SEQ ID No. 15. Based on these rice cDNA sequences, primers 5 and 6 were designed. Primer 5 is GCGGCGACCTCGCTCTACAC (SEQ ID No. 35). Primer 6 is TGTACGGGTACGCCGTGCA (SEQ ID No. 36). These primers were used to amplify a DNA fragment from rice genomic DNA using the procedure described above. A clean PCR product was directly sequenced using the individual PCR primers as sequencing primers. The sequence is as follows:

SEQ ID No. 27 is part of a xylanase inhibitor gene termed OSXI-01 from *Oryza sativa*:

GCGGCGACCTCGCTCTACACCATCCCCGTCAGGTACTACGACAACCTCGTCGTCGACCTCG
CCGGCCCGCTCGTCTGGTCGACGTGCGCCGCCGACCACCTGCCGGCGTCGCTGTCCTGCCA
GGACCCGACGTGCGTGGTCGCCAACGCGTACCGTGCTCCGACCTGCAAGGTCACCGGCGGC
GGCGGCGACTGCAGCAAGAACGTGTGCACGGCGTACCCGTACA.

SEQ ID No. 28 is part of a xylanase inhibitor protein encoded by SEQ ID No. 27:

AATSLYTIPVRYYDNLVVDLAGPLVWSTCAADHLPASLSCQDPTCVVANAYRAPTCKVTGG
GGDCSKNVCTAYPY.

Example 8. Maize protein encoding DNA sequences.

Primers 1 and 6 were also used to amplify a DNA fragment from *Zea mays* genomic DNA using identical procedures as described. A clean PCR product was directly sequenced using the individual PCR primers as sequencing primers. The sequence is as follows:

SEQ ID No. 29 is part of a xylanase inhibitor gene termed ZMXI-01 from *Zea mays*:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCGCTCGTCTGGTCCACGTGCCAGCGCGGCGATCTGCCGACAGATATCCCGTGCAGTAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCGCCCAGCTGCGGC
AGCGACAGGCACGACAAGCCGTGCACGGCGTACCCGTACA.

SEQ ID No. 30 is part of a xylanase inhibitor protein encoded by SEQ ID No. 29:

ATSLYTIPFHDGASLVLDVAGPLVWSTCQRGDLPTDIPCSSPTCLLANAYPAPGCPAPSCG
SDRHDKPCTAYPY.

Example 9. Barley protein encoding DNA sequences.

A BLAST search (TBLASTX 2.1.2, www.ncbi.nlm.nih.gov) in the database with non-human and non-mouse EST sequences using SEQ ID No. 6 (this patent application) produced significant alignment with a cDNA clone of barley:
IDENTIFIERS dbEST Id:5811794 EST name: HVSMEh0101D07f
GenBank Acc: BE602955

The present invention thus features a part of a HVXI variant having the amino acid sequence shown in SEQ ID No. 11:
AGFAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVGKQ
GSPAHYVSGTXIKVEDTRVPVPDRALVTGGVMLNTKLAYVLLRRDVYRPVVDAFTKALAAQ
HANGAPAARAVDPVAPFGLCYDAKTLGNNLGGYSVPNVVLALDGGGEWAMTGKNSMVDVKP
GX.

The present invention thus also features a part of a HVXI variant having the amino acid sequence shown in SEQ ID No. 12:
AGFAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVGKQ
GSPAHYVSGTXIKVEDTRVPVPDRALVTGGVMLNTKLAYVLLRRDVYRPVVDAFTKALAAQ
HANGALAARGVNPVAPFGLCYDAKTNGNNLGGYSVPNVVLALDGGGEWAMTGKNSMVDVKP
GX.

Or the present invention features a part of microheterogenic HVXI variants having the amino acid sequence shown in SEQ ID No. 13:
AGFAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVGKQ
GSPAHYVSGTXIKVEDTRVPVPDRALVTGGVMLNTKLAYVLLRRDVYRPVVDAFTKALAAQ
HANGAXAARXVXPVAPFGLCYDAKTXGNNLGGYSVPNVVLALDGGGEWAMTGKNSMVDVKP
GX, wherein **X** consists of an amino acid of the group P,A,D,L,G,N and C.

The obtained consensus sequence of HVXI (SEQ ID No. 14): (including ca. 48% of the coding sequence) is as follows:

GCGGGCTTCGCGGGCTCCGGCCTGGCGCTGCCGGCGCAGGTGGCGTCCGCGCAGAAGGTCT
CCCATCGGTTCCTCCTCTGCCTCCCCACGGGCGGCGCCGGCGTGGCCATCCTCGGCGGCGG
CCCGCTCCCGTGGCCGCAGTTCACGCAGTCCATGGCCTACACCCCGCTCGTCGGCAAGCAA
GGCAGCCCCGCGCACTACGTCTCGGGCACGTNCATCAAAGTCGAGGACACCCGCGTTCCCG
TTCCGGACCGCGCGCTCGTCACCGGGGGGGGTGATGCTCAACACGAAGCTGGCCTACGTCTT
GCTCCGCCGCGACGTGTACCGCCCGGTGGTGGACGCGTTCACCAAGGCCCTGGCGGCGCAG
CATGCCAACGGAGCGCCCGCGGCGCGCGCCGTGGACCCCGTGGCGCCGTTCGGGCTGTGCT
ACGACGCCAAGACGCTGGGCAACAACCTCGGCGGGTACTCGGTGCCCAACGTGGTGCTGGC
GCTCGACGGCGGGGGTGAATGGGCGATGACCGGGAAGAACTCGATGGTGGACGTCAAGCCG
GGGA.

Example 10. Oat protein encoding DNA sequences

Based on IDENTIFIERS dbEST Id: 5811794 EST name: HVSMEh0101D07f GenBank Acc: BE602955, primers 9 and 10 were designed. Primer 9 is TGGCGTCCGCGCAGAAGGTC (SEQ ID No. 44). Primer 10 is GCTTGACGTCCACCATCGAG (SEQ ID No. 45). These primers were used to amplify a DNA fragment from oat genomic DNA. The resulting PCR product was cloned according to procedures described above. The sequence is as follows:

SEQ ID No. 42 is part of a xylanase inhibitor gene termed ASXI-01 from *Avena sativa*:

TGGCGTCCGCGCAGAAGGTCGCCAAGAAGTTCCTCCTCTGCCTCTCCCGCGGCGGCGTGTA
CGGAGACGGCGTGGCCATCTTCGGCGGCGGCCCGCTCCACCTCACCGCGCAGCCGGAGACA
GACTACACGCAGTCCCTTGAGTACACGCCGCTCTTCACCAAAGAAGGCAACCCGGCGTACT
ACGTCTCGGTCAAGTCCATCGCGCTGGAGAACACCCCCGTCCCCGTCTCGACCCGCACGCT
CGACGCCGGCGGTGTGGTGCTCTGCACCAGGGTGCCATACACCTTTCTCCGCCCCGACGTG
TACCTCCCGTTCGCGGACGCGTTCCGCACGGCAATGAAGGCGCAGAAGGCGCAAGAAATGA
AGGCCGTGGCGCCATTCGGGCTGTGCTACAACACGTCGACGCTGGCCAACACGCGGCTCGG
GTACCTGGTGCCGACCGTGACGCTGGCGCTGGAAGGCGGGAAGAAGTGGACGATGACGGGC
GTCCACTCGATGGTGGACGTCAAGC.

The encoded xylanase inhibitor amino acid sequence is as follows:

SEQ ID No. 43 is part of a xylanase inhibitor encoded by SEQ ID No. 42:

ASAQKVAKKFLLCLSRGGVYGDGVAIFGGGPLHLTAQPETDYTQSLEYTPLFTKEGNPAYY
VSVKSIALENTPVPVSTRTLDAGGVVLCTRVPYTFLRPDVYLPFADAFRTAMKAQKAQEMK
AVAPFGLCYNTSTLANTRLGYLVPTVTLALEGGKKWTMTGVHSMVDVK.

Example 11. Recombinant expression of xylanase inhibitors

Materials and methods

Materials, strains and media

The TOPO TA Cloning Kit for Sequencing and the pBAD/TOPO ThioFusion Expression System were obtained from Invitrogen (Carlsbad, California, USA). The pMAL Protein Fusion and purification system was purchased from New England Biolabs (Beverly, MA, USA). The pHOS31 vector was derived from the pHEN1 plasmid [38] by insertion of the phage lambda cos site and a restriction site for *I-SceI* into the *AatII* site.

The following *E.coli* strains were used: TOP10 (genotype : F⁻ *mcrA* Δ(*mmr-hsd*RMS-*mcr*BC) Φ80*lacZ*Δ*M15* Δ*lac*X74 *recA1* *deoR* *ara*D139 Δ(*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *end*A1 *nup*G) (Invitrogen) and XL1-Blue MRF' (genotype : Δ(*mrcA*)183Δ(*mcrCB-hsdSMR-mmr*)173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* [F' *proAB* *lacI*^{q} *ZΔM15* *Tn10*(Tet^r)]) (Stratagene, La Jolla, CA, USA). *E.coli* strain TG1 (genotype : *supE* *hsdΔ5* *thi* Δ(*lac-proAB*) [F' *traD36* *proAB* *lacI*^{q} *lac* *ZΔM15*]) was used for expression experiments.

The LB-Amp medium (11) had the following composition : 10 g Tryptone Peptone (Difco), 5 g Selected Yeast Extract (Gibco BRL), 10 g NaCl (Acros Organics) and 100 µg/ml filter sterilised ampicillin. For agar medium, 15 g Select Agar (Gibco BRL) was added. One liter 2 x TYA contained 16 g Tryptone Peptone, 10 g Selected Yeast Extract, 5 g NaCl and 100 µg/µl filter sterilised ampicillin. In 2 x TYAG medium is 2 X TYA supplemented with 2% glucose.

Construction of the expression plasmids

The PCR 4-TOPO vector (Invitrogen) containing part of a xylanase inhibitor gene from *Triticum aestivum* cultivar Estica (example 5) was used as template for the construction of expression plasmids. Based on the sequence (SEQ ID No. 16), 2 primers were designed.
Forward primer 7 (SEQ ID No. 37) is
CCAAGATCT**CTGCCAGTTCTGGCACCTGTGACCAAAGATCCA**GCAACCTCCCTCTACAC
Reverse primer 8 (SEQ ID No. 38) is
CCTAGATCT**TTA**CAGGCCGCCGCAACCCGTAAAG. Both primers are positioned at the flanks of the sequence and contain at their 5′ end a *BglII* restriction site (underlined) plus 3 extra 5′ nucleotides. The forward primer (SEQ ID No. 37)contains a 3′ end corresponding to 5′ end of SEQ ID No. 16, plus a designed sequence encoding the N-terminal amino acids from mature TAXI (bold) as revealed by N-terminal sequencing of the native protein (SEQ ID No.1) and missing from the genomic sequence (SEQ ID No. 16). The reverse primer (SEQ ID No. 38) contains a 3′ end complementary to the 3′ end of the TAXI coding sequence including a stop codon (bold). Primers were ordered from Genset (Paris, France).

PCR reactions were performed in 50 µl using 0.05 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), commercially supplied buffer (Qiagen), 200µM of each dNTP, 1 µM of each primer and 200 ng of plasmid DNA. DNA amplification was carried out in an Eppendorf Mastercycler gradient (Hamburg, Germany) through an incubation step (15 min at 95°C), followed by 25 cycles of denaturation (1 min at 94°C), annealing (90 s at 57°C) and extension (2 min at 72°C). An additional extension step (20 min at 72°C) was added.

The resulting PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) and directly cloned into the pBAD/TOPO ThioFusion expression vector. The ligation mixture, containing 1 µl pBAD/Thio-TOPO vector (supplied in the kit), 3 µl MilliQ water and 2 µl purified PCR product,

was
incubated during 5 min at room temperature. The mixture was then placed on ice. Three µl of the ligation mixture was added to a vial of TOP10 One Shot Chemically Competent *E.coli* (supplied in the kit) previously mixed with 2 µl of 0.5M β-mercaptoethanol, and incubated during 30 min on ice. Subsequently, the cells were incubated during 30 s at 42°C and placed immediately back on ice. After addition of 250 µl SOC medium (supplied in the kit) and incubation in a shaker at 37°C, the transformed cells were plated on a selective (ampicillin) agar plate. Colonies were grown in liquid medium. Plasmid DNA of the resulting pBAD/Thio-TAXI transformants was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Insertions were verified by digestion with *Nru*I and by DNA sequencing using vector specific primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA). Sequencing gel was run on a 377 ABI PRISM DNA Sequencer (Applied Biosystems).

In a second step, the pBAD/Thio-TAXI vector was used as DNA source for the construction of two other expression plasmids, pHOS31-TAXI and pMAL-p2X-TAXI, respectively. Therefore, the inserted TAXI gene was cut out of the pBAD/Thio-TAXI vector with *Bgl*II . The vector was simultaneously digested with *Pvu*I to prevent self ligation and unwanted insertion in the other expression plasmids. The obtained restriction fragments were subsequently ligated overnight into a *Bgl*II cut, dephosphorylated pHOS31 vector, or a *BamH*I cut, dephosphorylated pMal-p2X plasmid. The ligation mixtures were transformed to electroporation competent XL1-Blue MRF' *E.coli* cells. The transformation mixture was spread on selective (ampicillin) agar plates. Positive clones were confirmed by restriction analysis with *Nru*I/ *BamH*I in the case of the pHOS31-TAXI constructs and with *Apa*I for the pMAL-p2X-TAXI constructs. The pMAL-p2X-TAXI construct is deposited with the Belgian Coordinated

Collection of Microorganisms under access number LMBP 4268.

Recombinant expression of TAXI from pBAD/Thio-TAXI

E.coli TOP10 cells containing the pBAD/Thio-TAXI vector were analysed for the expression of recombinant thioredoxin-TAXI fusion protein. An aliquot (100 µl) of an overnight grown culture was inoculated in 5 ml LB-Amp medium. The cultures were grown to mid-exponential phase (OD_{600nm} ~0.5) at 37°C in a shaker. Expression was induced by addition of 50 µl of a 2% arabinose solution. Cells were incubated at 37°C for another 4 hours. An aliquot (1 ml) was taken for SDS-PAGE analysis. Cells were pelleted and resuspended in 75 µl MilliQ water. The remaining cells were pelleted, resuspended in 2.5 ml lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10 mM imidazole, pH8) and incubated during 30 min on ice. Following 3 freeze-thaw cycles (at -70°C and room temperature), cells were sonicated on ice during 15 s. The resulting cleared lysate was used for measuring the endoxylanase inhibition activity.

Recombinant expression of TAXI from pHOS31-TAXI and pMAL-p2x-TAXI

Vectors, pHOS31-TAXI and pMAL-p2x-TAXI, and the corresponding parental plasmids were separately transformed into *E.coli* TG1 cells. Individual colonies were picked up, inoculated into 5 ml of 2 x TYAG and grown overnight at 37°C in a shaker. Subsequently, 500 µl of the overnight grown cultures was inoculated into 50 ml fresh 2 x TYAG medium. Cell cultures were grown at 37°C until an OD_{600nm} of ~0.6. Then, the cells were pelleted and resuspended in 50 ml 2 x TYA with 1mM IPTG. After 4 hours of incubation at 30°C in a shaker, the cells were harvested. The periplasmic protein fraction was isolated by cold osmotic shock. Therefore, cells were resuspended in 5 ml 30mM Tris-HCl, 20% sucrose, pH8. Following addition of 500 µl of a 10mM EDTA solution,

the cells were shaken at room temperature during 10 min. Subsequently, the cells were pelleted at 4°C, resuspended in 5 ml icecold 5mM $MgSO_4$ and placed on ice during 10 min while shaking. The cell suspensions were centrifuged and the resulting supernatants were used for SDS-PAGE analysis and measurement of endoxylanase inhibition activity.

Protein analysis

SDS-PAGE was performed with the Mini-PROTEAN II cell system (Biorad, USA) according to the manufacturer's instructions. Gels were stained with the SimplyBlue SafeStain following the basic protocol (Invitrogen, USA).

The endoxylanase inhibition activities were determined as described in the general experimental methods for examples 1 and 2. As little as 250 µl of an endoxylanase solution was added to an equal amount of sample. In the case of the pBAD/Thio-TAXI lysate, 50 µl of 0.5M AcOH was added to obtain a pH of about 5. The periplasmic protein fractions were used as such. The reaction was terminated by adding 2% Tris solution (5 ml) in stead of 10 ml of 1% Tris.

Purification of the recombinant MBP-TAXI fusion protein

Affinity chromatography was done to purify the MBP-TAXI fusion protein according to the method described in example 3. However, *A. niger* var. awamori endoxylanase purified as discussed in example 4 in stead of *B. subtilis* endoxylanase was immobilised. An aliquot (5 ml) of the periplasmic protein fraction of the pMAL-p2X-TAXI cells together with 800 µl protease inhibitor cocktail (1 Complete tablet (Boehringer Mannheim, Germany)and 400 µl pepstatin (5mM in ethanol) in 4 ml 200mM NaAc pH5 with 1.5M NaCl) was brought onto the column. Elution of MBP-TAXI was achieved with Tris buffer (250 mM) of pH8 in stead of pH10.

Results

Construction of the pBAD/Thio-TAXI vector and expression in *E.coli*

The PCR product, containing the complete coding sequence for a mature endoxylanase inhibitor protein from wheat and 2 additional *Bgl*II restriction sites at the flanks, was cloned in frame in a pBAD/Thio-TOPO vector under the control of the p_{BAD} promoter. DNA analysis of a retained clone revealed that there were 2 silent mutations.

The TAXI encoding DNA sequence, the flanking *Bgl*II restriction sites (bold) and the stop codon of the retained clone are represented in SEQ ID No. 39:

CCA**AGATCT**TTGCCAGTTCTGGCACCTGTGACCAAAGATCCAGCAACCTCCCTCTACACAA
TCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCGGCCCTCTCGTCTGGTCCAC
GTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAGCCCCACCTGCCTCCTCGCC
AACGCCTACCCCGCCCCGGGCTGCCCCGCTCCCAGCTGCGGCAGCGATAAGCACGACAAAC
CGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCGCCGCAGGGAGCCTCTCCCA
CACGAGATTCGTGGCCAACACCACCGACGGGAGCAAGCCGGTGAGCAAGGTGAACGTCGGG
GTCCTGGCAGCGTGCGCGCCGAGCAAGCTCCTAGCGTCGCTGCCCCGGGGCTCCACGGGCG
TGGCCGGGCTCGCGAACTCCGGCTTGGCGCTGCCGGCGCAGGTGGCATCCGCGCAGAAGGT
CGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCCTGGCGTGGCCATATTTGGCGGC
GGCCCGGTCCCGTGGCCGCAATTCACGCAGTCGATGCCTTACACGCCGCTCGTCACCAAGG
GCGGCAGCCCCGCGCACTACATCTCGGCCAGGTCCATTGTAGTGGGGGACACCCGCGTCCC
CGTACCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAGCACGAGGCTACCCTACGTC
TTGCTCCGCCCCGACGTGTACCGCCCGTTGATGGACGCGTTCACCAAGGCCCTGGCGGCGC
AGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGGCTGTGGCGCCGTTCGGGGTGTG
CTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGCGGTGCCCAACGTCCAGCTG
GGGCTCGATGGCGGCAGTGACTGGACGATGACCGGGAAGAACTCGATGGTGGACGTCAAGC
AAGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGGCGGCCGGCGACGGCAGGGC
GCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGTGCTCGACTTCGACATGGAG
AAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGTTGCGGCGGCCTGTAA**AGAT**
CTCCG.

Figure 21 shows the nucleotide and amino acid sequences of the PCR product. The nucleotides corresponding to TAXI SEQ ID No. 16 are indicated with a line above. The primers, containing a *BglII* restriction site (underlined), are represented in bold. The forward primer contains nucleotide sequences complementary to the 5' end of SEQ ID No. 16 and the N-terminal amino acids from mature TAXI missing from the genomic sequence. The reverse primer contains nucleotide sequences complementary to the 3' end of SEQ ID No. 16 and a stop codon. The TAXI amino acids are in italic.

Figure 22 shows the insertion of the PCR product in the pBAD/Thio-TOPO vector. The enterokinase recognition site and 3 C-terminal amino acids of the thioredoxin protein are also indicated.

Induction of the pBAD promoter is expected to lead to the cytoplasmic expression of a thioredoxin-TAXI fusion protein of about 55.7 kDa. SDS-PAGE analysis of the total protein fraction of the cells transformed with pBAD/Thio-TAXI showed that there was a prominent protein band of about 55.7 kDa. This protein was absent in the protein fraction of the cells containing empty pBAD/Thio vector.

Figure 23 A represents the recombinant thioredoxin-TAXI fusion protein.

Figure 24 shows the SDS-PAGE profiles of the total cell extracts of the pBAD/Thio cells and the pBAD/Thio-TAXI cells (lanes 1 and 2 respectively). The low molecular mass markers are situated in lane 5. The size of the markers is indicated on the right.

Total cell lysates were used to measure the endoxylanase inhibition activity against *Bacillus subtilis* endoxylanase. No endoxylanase inhibition activity was measured in the extract containing the thioredoxin-TAXI fusion protein and the control containing thioredoxin. It appeared that the thioredoxin-TAXI producing cells contained

large inclusion bodies, as seen by phase contrast microscopy, suggesting improper folding and aggregation of the recombinant protein.

Construction of the pHOS31-TAXI and the pMAL-p2X-TAXI vectors and expression in TG1 *E.coli*

The TAXI gene (SEQ ID No. 39) was cut out the pBAD/Thio-TAXI vector with the flanking *Bgl*II restriction sites and cloned in frame into a pHOS31 and a pMAL-p2X vector.

The pHOS31 and the pMAL-p2X vector are under the control of respectively a P_{lacz} promoter and a P_{tac} promoter and can be induced by adding IPTG. The presence of the *pelB* leader sequence and the *malE* signal sequence in respectively pHOS31 and pMAL-p2X, allow fusion proteins to be exported to the periplasm. In the case of the pHOS31-TAXI vector, a ca. 40 kDa TAXI protein will be expressed. The pMAL-p2X-TAXI construct leads to a MBP (maltose binding protein)-TAXI fusion protein of about 82 kDA. Both plasmids, pHOS31-TAXI and pMAL-p2X-TAXI, and the corresponding parental plasmids, were seperately transformed to *E.coli* TG1 cells to perform the expression experiments.

Figure 25 and 26 show the insertion of the *Bgl*II cut PCR product in respectively the *Bgl*II and the *BamHI* restriction site of respectively the pHOS31 vector and the pMAL-p2X vector. The C-terminal amino acids of the *pelB* leader sequence respectively the *malE* signal sequence together with the 'linker' amino acids are also represented.

Figure 23 B shows the recombinant TAXI protein as expressed by the pHOS31-TAXI plasmid.

Figure 23 C shows the recombinant MBP-TAXI protein as expressed by the pMAL-p2X-TAXI plasmid.

SDS-PAGE analysis of the periplasmic fractions of pHOS31-TAXI showed no clear TAXI protein band. However, the ca. 82 kDa MBP-TAXI fusion protein was prominent.

Figure 24 shows the SDS-PAGE profiles of the periplasmic protein extract with the ca. 82 kDa MBP-TAXI fusion protein or the ca. 50.8 kDa MBP control protein (lanes 4 and 3 respectively). The molecular mass markers are in lane 5 and represented on the right.

The periplasmic protein fractions of the pHOS31-TAXI and the pMAL-p2X-TAXI *E.coli* cells were used to measure the endoxylanase inhibition activity against *A.niger* and *B.subtilis* endoxylanase. Both the pHOS31-TAXI fraction and the pMAL-p2X-TAXI fraction showed endoxylanase inhibition activity against *A.niger* (96% and 99%, respectively) and *B.subtilis* (81% and 88%, respectively) endoxylanase. This indicates that a recombinant TAXI I protein was produced.

Purification of recombinant MBP-TAXI fusion protein

SDS-PAGE analysis showed that the ca. 82 kDa MBP-TAXI fusion protein was efficiently purified from the periplasmic protein fraction by affinity chromatography on immobilised *A.niger* endoxylanases. The recombinant TAXI protein has molecular form A as under reducing conditions no dissociation of the protein was noticed.

Figure 27 shows the SDS-PAGE profile of the ca. 82 kDa purified MBP-TAXI fusion protein (lane 1).The molecular mass markers are in lane 2 and indicated on the right.

Demonstration of endoxylanase inhibition activity by extracts from *E.coli* harboring the recombinant TAXI sequences was the final proof that the gene sequences identified do encode the inhibitor activity. The cloned gene can be efficiently expressed in *E.coli* into active form when secreted into the periplasm with or without a fusion protein. TAXI produced in the cytoplasm appeared inactive, most

probably due to failure of accurate disulphide bond formation and/or folding into the native state. Recombinant TAXI is active as a single chain (form A) protein.

General conclusion

As exemplified, two general methods can be used for the purification of endoxylanase inhibitors from wheat flour, and barley whole meal. Very likely, they are both also applicable for rye, durum wheat and a broad spectrum of other plant sources.

A first method comprises several (four) separations on cation exchange columns and one separation on a gel filtration column to obtain pure inhibitor. A second method comprises two purification steps, one with cation exchange chromatography and another with affinity chromatography, to obtain a pure inhibitor sample containing one or more endoxylanase inhibitors. These inhibitors can at least partially be separated from one another by further fractionations with high resolution cation exchange chromatography. This second approach to purify endoxylanase inhibitors is a highly efficient purification method since using said method we could isolate inhibitor proteins from wheat, rye and barley with large structural similarity (e.g. amino acid sequence and SDS-PAGE profile) and similarity in activity pattern as described above, as well as other endoxylanase inhibitor proteins.

In the case of wheat flour (var. Soissons) two endoxylanase inhibitors, TAXI I and TAXI II, could be purified with the first purification method and were partially characterised. Depending on the immobilised endoxylanase and the elution conditions, the second purification method allowed for the isolation of at least five inhibitors (TAXI) as well as for the isolation of other xylanase inhibitors (non-TAXI) from commercially available

wheat flour (likely a mixture of different wheat varieties).

In the case of barley whole meal (var. Hiro) one endoxylanase inhibitor, HVXI, could be purified with the first purification method and was partially characterised. However, we have indications that at least one additional endoxylanase inhibitor may be present in barley as well. Using the second method, the purification of HVXI was also successful.

In the case of rye flour (var. Halor) the second purification method resulted in several endoxylanase inhibitors (SCXI I-V), with highly similar specificities and characteristics.

The wheat, barley and rye endoxylanase inhibitors under consideration are all characterised by similar molecular masses (ca. 40.0 kDa) and structures. They occur in two different forms, i.e. proteolytically modified ones and non-modified ones. The modified forms dissociate in two polypeptides (ca. 30.0 and ca. 10.0 kDa) upon reduction with β-mercaptoethanol. Neither of the inhibitors are glycosylated. TAXI I has a pI of 8.8 where as TAXI II and HVXI have pI values of 9.3 or higher. The N-terminal amino acid sequences of these three inhibitors show a high degree of mutual identity, especially those of the ca. 40.0 kDa polypeptides, and are not described as such for proteins of other sources.

Despite the difference in pI, TAXI I and HVXI, have similar effects on the five endoxylanases mentioned above. In contrast to TAXI I and HVXI, TAXI II has only little if any inhibition activity against the *A. niger* endoxylanase, but it similarly affects the other four endoxylanases.

Studies on the inhibition type of TAXI I and TAXI II unexpectedly show that the type of inhibition depends on the endoxylanase used. The *A. niger* endoxylanase is inhibited by TAXI I by blocking the active site, i.e. TAXI I

competes with arabinoxylan, and in the case of the *B. subtilis* endoxylanase, both TAXI I or TAXI II and arabinoxylan can bind and this independent of the binding order, i.e. TAXI I and TAXI II do not compete with arabinoxylan. Because of their similar endoxylanase inhibition profiles, it is not unreasonable to assume that HVXI inhibits the *A. niger* and *B. subtilis* endoxylanases in a manner analogous to that of TAXI I.

We also document for the first time a new technique for the purification of endoxylanases from commercially available enzyme preparations based on affinity chromatography with an immobilised cocktail of 'TAXI'-like endoxylanase-inhibitors.

Furthermore, the invention features not only amino acid sequences of endoxylanase inhibitors, but also corresponding encoding polynucleotide sequences and variants, homologues or fragments thereof.

A TAXI gene was cloned and recombinant active TAXI proteins including a fusion protein were produced by *E.coli*.

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CLAIMS

1. An isolated nucleic acid molecule encoding an inhibitor, characterised in that said inhibitor inhibits cellulase, endoxylanase, β-glucanase, β-xylosidase, α-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes.
2. The isolated nucleic acid molecule of claim 1, encoding a xylanase inhibitor or a variant, homologue or fragment thereof.
3. The isolated nucleic acid molecule of claim 1 or 2, comprising a first polynucleotide sequence 70%, 80%, 90% or 95% or more identical to a second polynucleotide selected from the group consisting of: (a) a polynucleotide sequence encoding amino acids 1 to 185 of SEQ ID No. 11; (b) a polynucleotide sequence encoding amino acids 1 to 185 of SEQ ID No. 12; (c) a polynucleotide sequence encoding amino acids 1 to 185 of SEQ ID No.13; (d) a polynucleotide sequence encoding amino acids 1 to 381 of SEQ ID No. 7; (e) a polynucleotide sequence encoding amino acids 1 to 381 of SEQ ID No. 8; (f) a polynucleotide sequence encoding amino acids 1 to 381 of SEQ ID No. 9; (g) a polynucleotide sequence encoding amino acids 1 to 370 of SEQ ID No. 19; (h) a polynucleotide sequence encoding amino acids 1 to 370 of SEQ ID No. 20; (i) a polynucleotide sequence encoding amino acids 1 to 196 of SEQ ID No. 21; (j) a polynucleotide sequence encoding amino acids 1 to 277 of SEQ ID No. 22; (k) a polynucleotide sequence encoding amino acids 1 to 102 of SEQ ID No. 25; (l) a polynucleotide sequence encoding amino acids 1 to 102 of SEQ ID No. 26; (m) a polynucleotide sequence encoding amino acids 1 to 75 of SEQ ID No. 28; (n) a polynucleotide sequence encoding amino acids 1 to 74 of SEQ ID No. 30; (o) a polynucleotide sequence encoding amino acids 1 to 286 of SEQ ID No .41 and (p) a polynucleotide sequence encoding amino acids 1 to 170 of SEQ ID No. 43.
4. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (a).
5. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (b).
6. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (c).

7. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (d).
8. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (e).
9. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (f).
10. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (g).
11. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (h).
12. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (i).
13. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (j).
14. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (k).
15. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (l).
16. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (m).
17. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (n).
18. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (o).
19. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (p).
20. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (a).
21. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (b).
22. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (c).

23. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (d).
24. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (e).
25. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (f).
26. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (g).
27. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (h).
28. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (i).
29. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (j).
30. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (k).
31. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (l).
32. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (m).
33. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (n).
34. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (o).
35. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (p).
36. The isolated nucleic acid molecule of claim 1 or 2 selected from the group consisting of :

(a) an isolated polynucleotide comprising a polynucleotide sequence that is at least 70%, 80%, 90% or 95% or more identical to polynucleotide sequence SEQ ID No. 10, SEQ ID No. 14., SEQ ID No. 15., SEQ ID No 16., SEQ ID No. 17., SEQ ID No. 18., SEQ ID No. 23., SEQ ID No. 24., SEQ ID No. 27., SEQ ID No 29., SEQ ID No. 39., SEQ ID

No. 40. or SEQ ID No. 42. over the entire length of SEQ ID No. 10., SEQ ID No. 14., SEQ ID No. 15., SEQ ID No 16., SEQ ID No. 17., SEQ ID No. 18., SEQ ID No. 23., SEQ ID No. 24., SEQ ID No. 27., SEQ ID No 29., SEQ ID No. 39., SEQ ID No. 40. or SEQ ID No. 42.

(b) an isolated polynucleotide which is the polynucleotide of SEQ ID No. 10., SEQ ID No. 14., SEQ ID No. 15., SEQ ID No 16., SEQ ID No. 17., SEQ ID No. 18., SEQ ID No. 23., SEQ ID No. 24., SEQ ID No. 27., SEQ ID No 29., SEQ ID No. 39., SEQ ID No. 40. or SEQ ID No. 42.

(c) an isolated polynucleotide obtainable by screening an appropriate library under stringent hybridisation conditions with a probe having the sequence of SEQ ID No. 10, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 27, SEQ ID No 29, SEQ ID No. 39, SEQ ID No. 40 or SEQ ID No. 42 or a fragment thereof.

(d) An isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide that is at least 70%, 80%, 90% or 95% or more identical to the amino acid sequence over its entire length of an inhibitor of cellulolytic, xylanolytic or β-glucanolytic enzymes, with the amino acid sequence SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28. or SEQ ID No.30. ., SEQ ID No. 41. or SEQ ID No. 43.

(e) An isolated polynucleotide comprising a polynucleotide sequence encoding an inhibitor of cellulolytic, xylanolytic or β-glucanolytic enzymes, with the amino acid sequence SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30., SEQ ID No. 41 or SEQ ID No. 43.

(f) A nucleic acid sequence encoding the complete amino acid sequence encoded by the DNA contained in LMBP deposit at the Belgian Coordinated Collection of Microorganisms with deposit number LMBP 4268.

(g) An isolated polynucleotide encoding a protein expressed by the xylanase inhibitor gene

contained in plants; and

(h) A polynucleotide sequence complementary to said isolated polynucleotide of (a), (b), (c), (d), (e), (f) or (g).

37. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 10.

38. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 14.

39. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 15.

40. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 16.

41. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 17.

42. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 18.

43. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 23.

44. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 24.

45. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 27.

46. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 29.

47. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 39.

48. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 40.

49. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 42.

50. The polynucleotide of any of the claims 1 to 49, wherein the polynucleotide is DNA.

51. The polynucleotide of any of the claims 1 to 49, wherein the polynucleotide is genomic DNA.

52. A polynucleotide probe or primer comprising at least 15 contiguous nucleotides or the polynucleotide of any of the claims 1 to 49.
53. A polynucleotide sequence according to claim 1 to 51 operably linked to a promoter.
54. A recombinant DNA construct comprising at least one of the polynucleotide sequences of claim 1 to 51.
55. The transcribed RNA product of the polynucleotide of any of the claims 1 to 51.
56. An RNA molecule or a fragment thereof which is antisense in relation to the RNA product of claim 55 and is capable of hybridising thereto.
57. A vector comprising the polynucleotide sequence according to any of the claims 1 to 51.
58. An expression system transformed with a DNA molecule according to any of the claims 1 to 51.
59. An expression system as in claim 58 and deposited with the Belgian Coordinated Collection of Microorganisms, under access number LMBP 4268.
60. A host organism transformed with the DNA molecule according to any of the claims 1 to 51.
61. The host organism of claim 60, wherein the DNA molecule according to any of the claims 1 to 51 is operably associated with a heterologous regulatory sequence.
62. The host organism of claim 60 or claim 61, wherein said host organism is a microorganism, plant, plant tissue or plant cell.
63. Method for transforming microorganisms, plants tissues or plant cells by the polynucleotides of claim 1 to claim 51, wherein the activity of the inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, α-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes is reduced.
64. Method according to claim 63, characterised in that the reduced activity of said inhibitor according to the invention is obtained by reduction of its expression.
65. Method according to claim 63, characterised in that the activity of said inhibitor is reduced by blocking the inhibitor function.
66. Method for transforming microorganisms, plants tissues or plant cells by polynucleotides of claim 1 to claim 51, wherein the activity of the inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes is increased.
67. Method according to claim 66, characterised in that increased activity of the inhibitor

according to the invention is obtained by an increase of its expression.

68. Method according to claim 66, characterised in that the activity of the inhibitor is increased by activating the inhibitor function.

69. The method of claim 66 or claim 67 for producing a recombinant protein, which is a proteinic or glycoproteinic inhibitor of cellulolytic, xylanolytic and/or β-glucanolytic enzymes, comprising: culturing a host organism comprising a nucleotide molecule according to any of the claims 1 to 51, under conditions suitable to produce said protein by said nucleic acid and recovering said protein.

70. The method of claim 69 for producing a recombinant protein, which is a proteinic or glycoproteinic inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes or fragment thereof comprising culturing the host organism of claim 60 to claim 62, whereby the recombinant protein is produced and recovering said recombinant protein.

71. The method of claim 69 or 70, comprising a method for separation or isolation of said recombinant inhibitor comprising screening the inhibition activity by using two or more enzymes during the separation or isolation steps that allow to distinguish inhibitors of different specificity.

72. The method as in claim 71 wherein the enzymes used are endoxylanases.

73. The method as in claim 72 wherein the enzymes used comprise a *Bacillus subtilis* and/or an *Aspergillus niger* endoxylanase.

74. The method as in any of the claims 69 to 73 comprising a cation-exchange chromatographic step and/or an anion-exchange chromatographic step.

75. The method as in any of the claims 69 to 71 comprising separating and/or isolating said recombinant inhibitor comprising an affinity chromatographic step with immobilised enzymes and/or antibodies against said recombinant polypeptides or fragments thereof.

76. The method as in claim 75 where the immobilised enzyme is an endoxylanase and the antibody is an antibody against said recombinant inhibitor.

77. The method as in any of the claims 75 and 76 where the immobilised endoxylanases are those of *B. subtilis* and/or *A. niger*.

78. The method as in any of the claims 75 to 77 comprising an additional cation exchange chromatographic step and/or an anion-exchange chromatographic step.

79. The method as in any of the claims 75 to 78 comprising screening the inhibition activity by using two or more enzymes during the separation and/or isolation steps that allow to distinguish inhibitors of different specificity.

80. A recombinant protein, recombinant glycoprotein or recombinant polypeptide or fragments thereof, which is an inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes or fragment thereof produced by the process of any of the claims 69 to 79.

81. A recombinant protein, recombinant glycoprotein or recombinant polypeptide or fragments thereof, which is an inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes or fragment thereof, coded by a nucleotide molecule according to any of the claims 1 to 51.

82. The recombinant protein of claim 80 or claim 81, which is a xylanase inhibitor.

83. The inhibitor of any of the claims 80 to 82, with an amino acid sequence 70%, 80%, 90% or 95% or more identical to SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43.

84. The inhibitor of any of the claims 80 to 83, characterised in that it has to capacity of only partially inactivating its ligand.

85. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants and/or the plant materials according to claim 69 for formation of endoxylanase-inhibitor complex wherein the inhibitor mimics the normal substrate or wherein it binds in a way that it does not prevent binding of the normal substrate.

86. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants and/or the plant materials according to claim 62 for screening endoxylanases that are totally, less or not inhibited by said inhibitor(s) or for modifying endoxylanases in such way that they are totally, less or not inhibited by said inhibitor(s).

87. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for reducing syruping

in refrigerated dough compositions, said refrigerated dough compositions comprising flour and water.

88. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for affecting the relative affinity and/or relative hydrolysis specificity and/or relative hydrolysis rate versus water-extractable and/or water-unextractable arabinoxylans of endoxylanases such as by the formation of an endoxylanase/inhibitor complex.

89. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants and/or the plant materials according to claim 62 for improving the malting of cereals such as barley, sorghum and wheat and/or the production of beer.

90. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving the production and/or quality of baked or extruded cereal products chosen among the group consisting of straight dough, sponge dough, Chorleywood bread, breakfast cereals, biscuits, pasta and noodles.

91. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant tissues according to claim 62, for improving animal feedstuff efficiency.

92. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving the production of starch derived syrups, sorbitol, xylose and/or xylitol.

93. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for wheat gluten-starch separation and production.

94. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving maize processing.

95. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving plant disease resistance.

96. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving

nutraceutical and/or pharmaceutical applications.

97. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving paper and pulp technologies.

98. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for purifying endoxylanases.

99. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for purifying endoxylanases in a process comprising affinity chromatography.

100. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for purifying endoxylanases in a process comprising affinity chromatography on N-hydroxysuccinimide(NHS)-activated Sepharose® 4 Fast Flow.

101. The inhibitor as in any of the claims 80 to 84, characterised in that said inhibitor is immobilised on an affinity chromatography support.

102. A method for producing protein isolates, comprising the steps of complexing said protein with or binding said protein to said immobilised inhibitor of claim 101, and subsequent disassociation of said complex to produce the protein isolate.

103. Depletion of xylanase inhibitor ligands in a medium or mixture of compounds by complexing said xylanase inhibitor ligands with said immobilised inhibitor of claim 101, and subsequent collecting said xylanase inhibitor ligands depleted medium.

104. The method of claim 102 or claim 103, wherein said xylanase inhibitor ligands is a xylanase.

105. A preparation containing the xylanase inhibitor ligands depleted fraction obtainable by the method of claim 102 or claim 103.

106. The preparation of claim 105, wherein the xylanase inhibitor ligands depleted fraction is from a mixture of enzymes.

107. The preparation of claim 106, for modification or degradation of beta-glucan containing materials.

108. The preparation of any of the claims 105 to 107, for further isolating selected xylanases that are not inhibited by a selected xylanase inhibitor.

123. A proteinic or glycoproteinic inhibitor of cellulolytic, xylanolytic and/or β-glucanolytic enzymes, obtainable by the method of any of the claims 114 to 122, characterised by having a marker whose amino acid sequence has more than 70% homology with SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43.

124. Inhibitor as in claim 123, characterised in that the marker is the N-terminal amino acid sequence of the protein or glycoprotein.

125. Inhibitor as in claim 123 having a marker for which the amino acid sequence has more than 85% homology with SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43.

126. Inhibitor as in claim 125, characterised in that the marker is the N-terminal amino acid sequence of the protein or glycoprotein.

127. Inhibitor as in claim 123, having a marker whose amino acid sequence is identical to SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43., or a variant, homologue or fragment thereof.

128. Inhibitor as in claim 127, characterised in that the marker is the N-terminal amino acid sequence of the protein or glycoprotein.

129. Inhibitor as in any of the claims 123 to 128, characterised in that said inhibitor inhibits cellulase, endoxylanase, β-glucanase, β-xylosidase, α-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes.

130. Inhibitor as in any of the claims 123 to 129 or fractions thereof, characterised in that it is obtainable from plant material.

109. Preparations of any of the claims 105 to 107 characterised in that they contain xylanases that are not inhibited by selected xylanase inhibitors for degradation, modification or degradation of arabinoxylans in the presence said selected xylanase inhibitors.
110. An isolated antibody or fragment thereof, which specifically binds to said inhibitor of claim 80 to claim 84.
111. A compound, which modulates said inhibitor of any of the claims 80 to 84.
112. A compound according to claim 111 which antagonises or selectively antagonises said inhibitor of claim 80 to claim 84.
113. A compound according to claim 111, which agonises said inhibitor of claim 80 to claim 84.
114. A method for separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes comprising screening the inhibition activity by using two or more enzymes during the separation and/or isolation steps that allow to distinguish inhibitors of different specificity.
115. A method as in claim 114, wherein the enzymes used are endoxylanases.
116. A method as in claim 114 or claim 115, wherein the enzymes used comprise a *Bacillus subtilis* and/or an *Aspergillus niger* endoxylanase.
117. A method as in any of the claims 115 to 116, comprising a cation-exchange chromatographic step and/or an anion-exchange chromatographic step.
118. A method for separation and/or isolation of cellulolytic, xylanolytic and/or β-glucanolytic enzymes comprising an affinity chromatographic step with immobilised enzymes and/or antibodies against inhibitors.
119. A method as in claim 118 where the immobilised enzyme is an endoxylanase and the antibody is an antibody against the endoxylanase inhibitor.
120. A method as in any of the claims 118 and 119 where the immobilised endoxylanases are those of *B. subtilis* and/or *A. niger*.
121. A method as in any of the claims 118 to 120 comprising an additional cation-exchange chromatographic step and/or an anion-exchange chromatographic step.
122. A method as in any of the claims 118 to 121 comprising screening the inhibition activity by using two or more enzymes during the separation and/or isolation steps that allow to distinguish inhibitors of different specificity.

131. Inhibitor as in claim 129, characterised in that said plant material is selected from the group consisting of cereals, cereal grains, cereal germs or cereal flours from wheat, durum wheat, rye, triticale, barley, sorghum, oats, maize or rice.
132. Inhibitor as in any of the claims 123 to 130, characterised in that it is obtainable from microorganisms or fractions thereof.
133. Inhibitor as in any of the claims 123 to 132, characterised in that it is an endoxylanase inhibitor.
134. Inhibitor as in claim 133, characterised in that it is a water-soluble species.
135. Inhibitor as in any of the claims 123 to 134, characterised in that said protein or glycoprotein is selected from the group comprising proteins or glycoproteins having a molecular weight typically between 40 kDa and 43 kDa, proteins or glycoproteins having a molecular weight of typically 30 kDa and proteins or glycoproteins having a molecular weight of typically 10 kDa.
136. Inhibitor as in any of the claims 123 to 135, characterised in that said protein or glycoprotein typically has a molecular weight between 40 kDa and 43 kDa and a pI greater than 7 or about 7.
137. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122, for formation of endoxylanase-inhibitor complex wherein the inhibitor mimics the normal substrate or wherein it binds in a way that it does not prevent binding of the normal substrate.
138. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122, for screening endoxylanases that are totally, less or not inhibited by said inhibitor(s) or for modifying endoxylanases in such way that they are totally, less or not inhibited by said inhibitor(s).
139. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for reducing syruping in refrigerated dough compositions, said refrigerated dough compositions comprising flour and water.
140. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for affecting the relative affinity and/or relative hydrolysis specificity and/or relative hydrolysis rate versus water-extractable and/or water-unextractable arabinoxylans of endoxylanases such as by the formation of an endoxylanase/inhibitor complex.

141. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving the malting of cereals such as barley, sorghum and wheat and/or the production of beer.
142. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving the production and/or quality of baked or extruded cereal products chosen among the group consisting of straight dough, sponge dough, Chorleywood bread, breakfast cereals, biscuits, pasta and noodles.
143. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving animal feedstuff efficiency.
144. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving the production of starch derived syrups, sorbitol, xylose and/or xylitol.
145. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for wheat gluten-starch separation and production.
146. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving maize processing.
147. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving plant disease resistance.
148. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving nutraceutical and/or pharmaceutical applications.
149. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving paper and pulp technologies.
150. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for purifying endoxylanases.
151. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for purifying endoxylanases in a process comprising affinity chromatography.
152. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for purifying endoxylanases in a process

comprising affinity chromatography on N-hydroxysuccinimide(NHS)-activated Sepharose® 4 Fast Flow.

153. The inhibitor as in any of the claims 123 to 136, characterised in that said inhibitor is immobilised on an affinity chromatography support.

154. A method of producing protein isolates, comprising the steps of complexing said protein with or binding said protein to said immobilised inhibitor of claim 153, and subsequent disassociation of said complex to produce the protein isolate.

155. Depletion of xylanase inhibitor ligands in a medium or mixture of compounds by complexing said xylanase inhibitor ligands with said immobilised inhibitor of claim 153, and subsequent collecting said xylanase inhibitor ligands depleted medium.

156. The method of claim 154 or 155, wherein said xylanase inhibitor ligands is a xylanase.

157. A preparation containing the xylanase inhibitor ligands depleted fraction obtainable by the method of claim 155 or claim 156.

158. The preparation of claim 157, wherein the xylanase inhibitor ligands depleted fraction is from a mixture of enzymes.

159. The preparation of claim 158, for modification or degradation of beta-glucan containing materials.

160. The preparation of claim 157 to 159, for further isolating selected xylanases that are not inhibited by a selected xylanase inhibitor.

161. Preparations of claim 157 to 159 characterised in that they contain xylanases that are not inhibited by selected xylanase inhibitors for degradation, modification or degradation of arabinoxylans in the presence of said selected xylanase inhibitors.

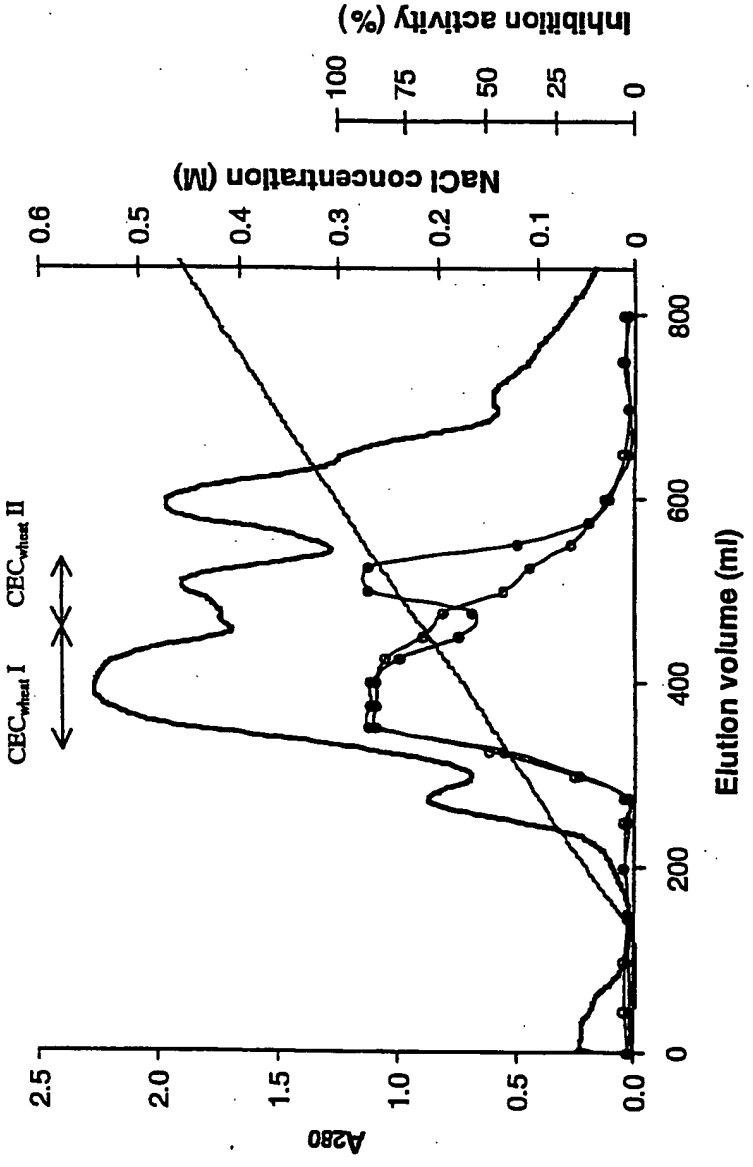
Inhibition activity (%)
100
75
50
25
0
NaCl concentration (M)
0.6
0.5
0.4
0.3
0.2
0.1
0
A280
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
Elution volume (ml)
CEC$_{wheat}$ I
CEC$_{wheat}$ II

Figure 1

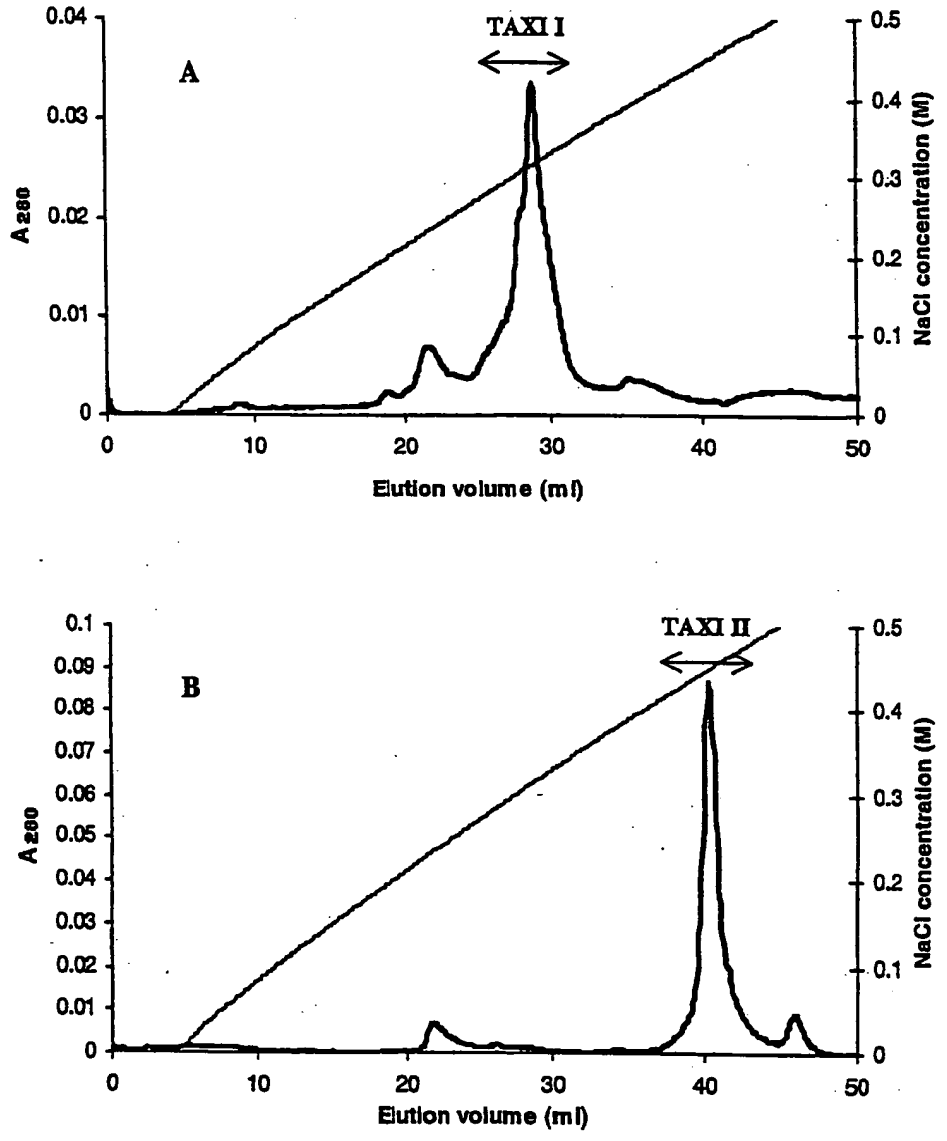
A
TAXI I
A280
NaCl concentration (M)
Elution volume (ml)
B
TAXI II
A280
NaCl concentration (M)
Elution volume (ml)

Figure 2

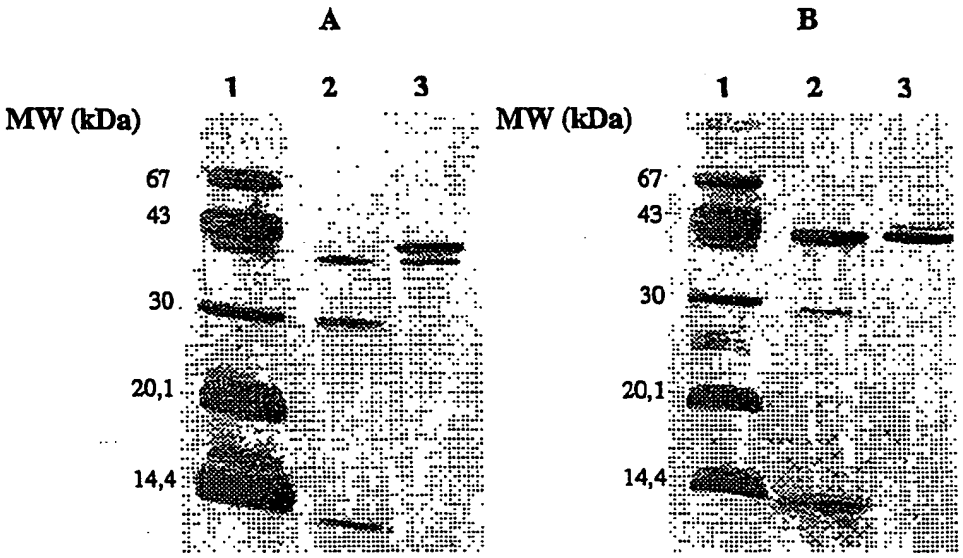

Figure 3

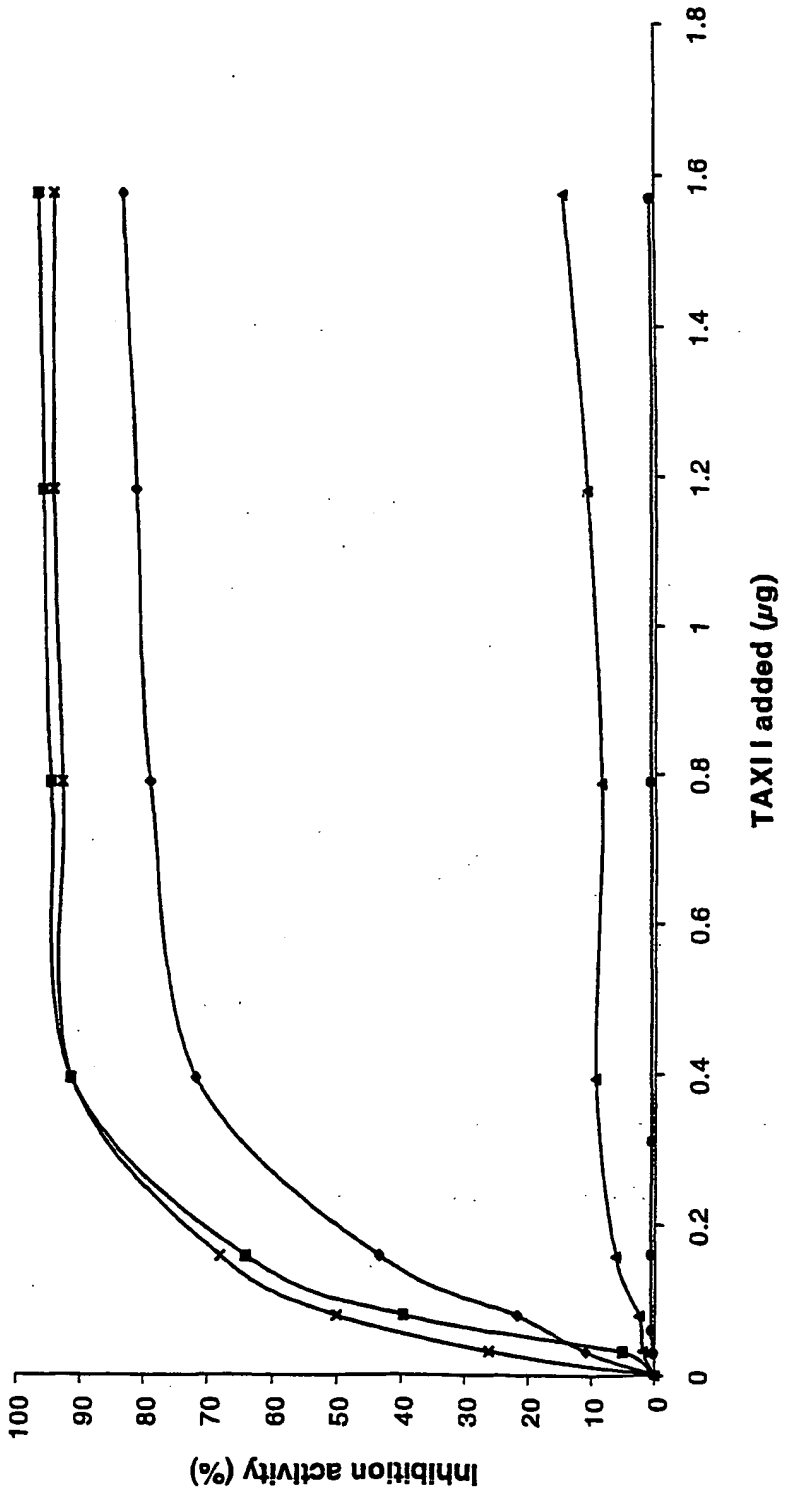
Inhibition activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
TAXII added (µg)

Figure 4

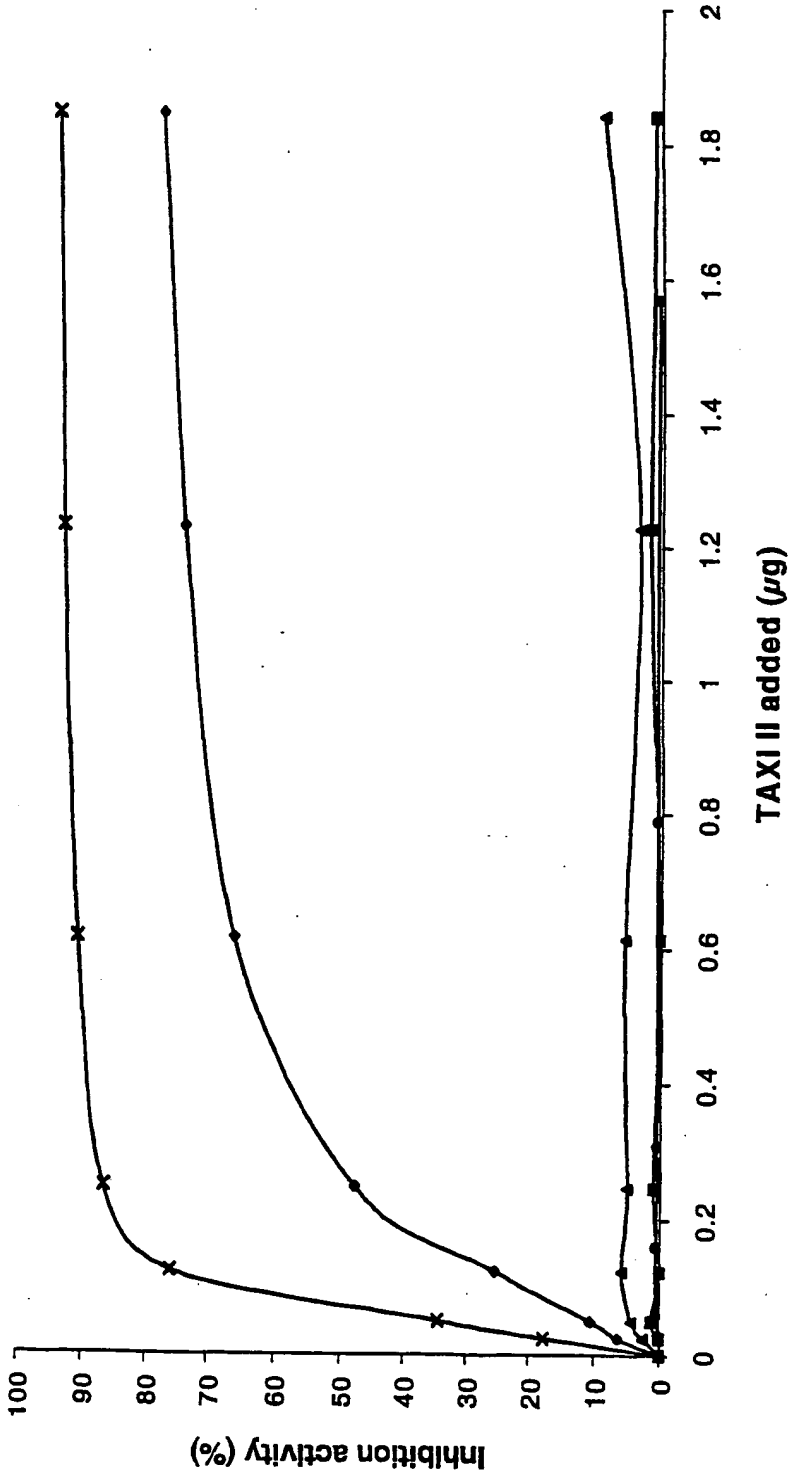
Inhibition activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
TAXI II added (µg)

Figure 5

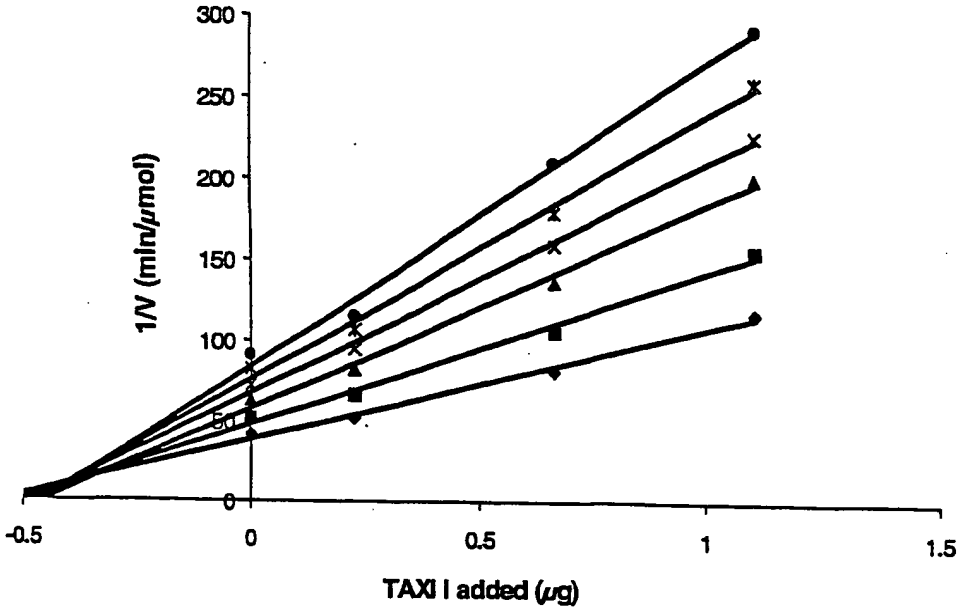

Figure 6

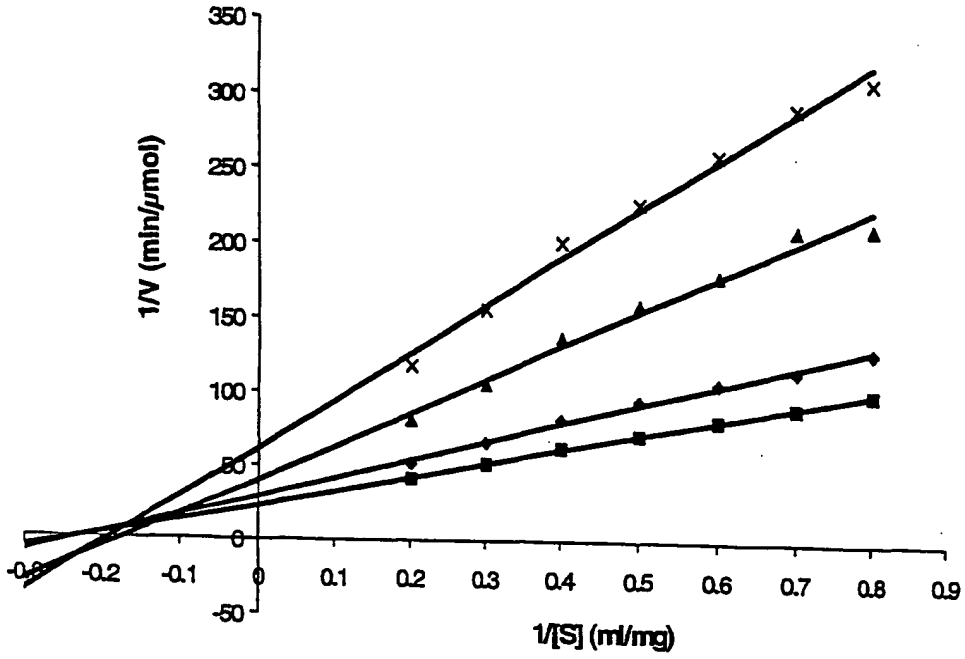
350
300
250
200
150
100
50
0
-50
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/V (mln/µmol)
1/[S] (ml/mg)

Figure 7

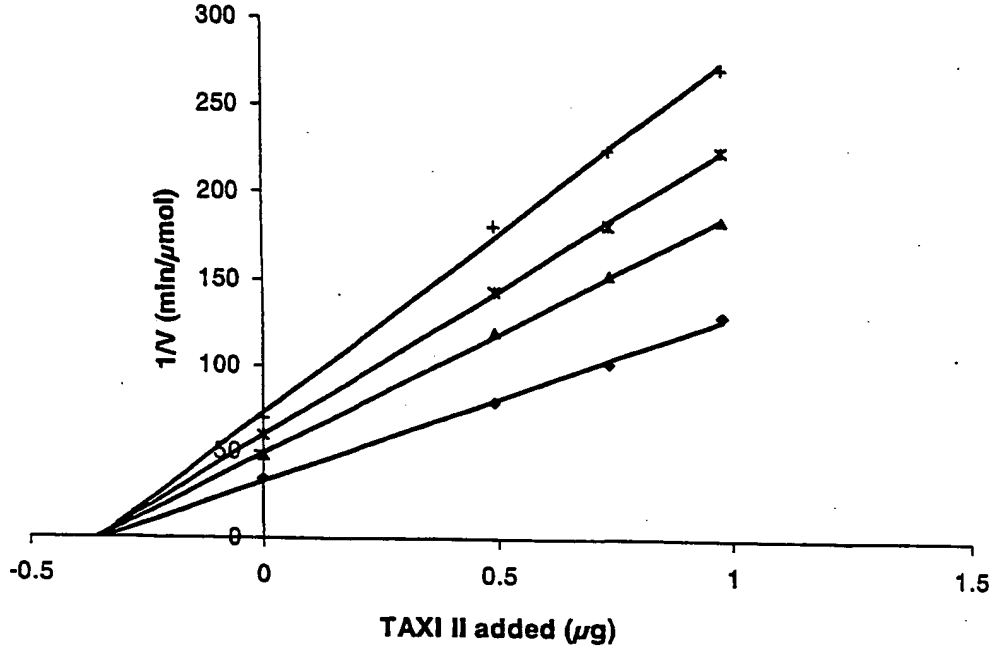

Figure 8

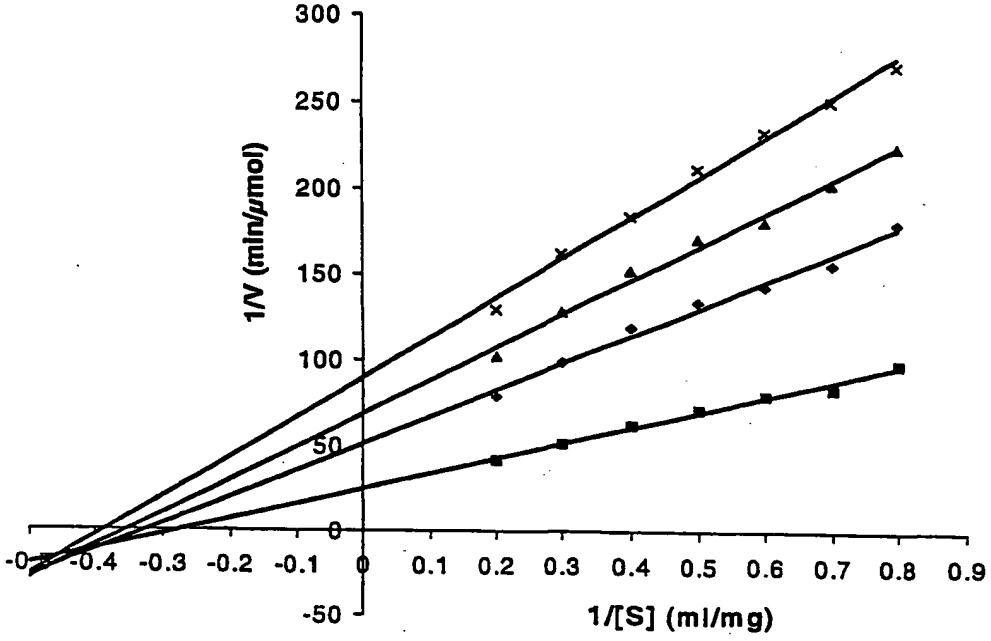
300
250
200
150
100
50
0
-50
1/V (min/µmol)
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/[S] (ml/mg)

Figure 9

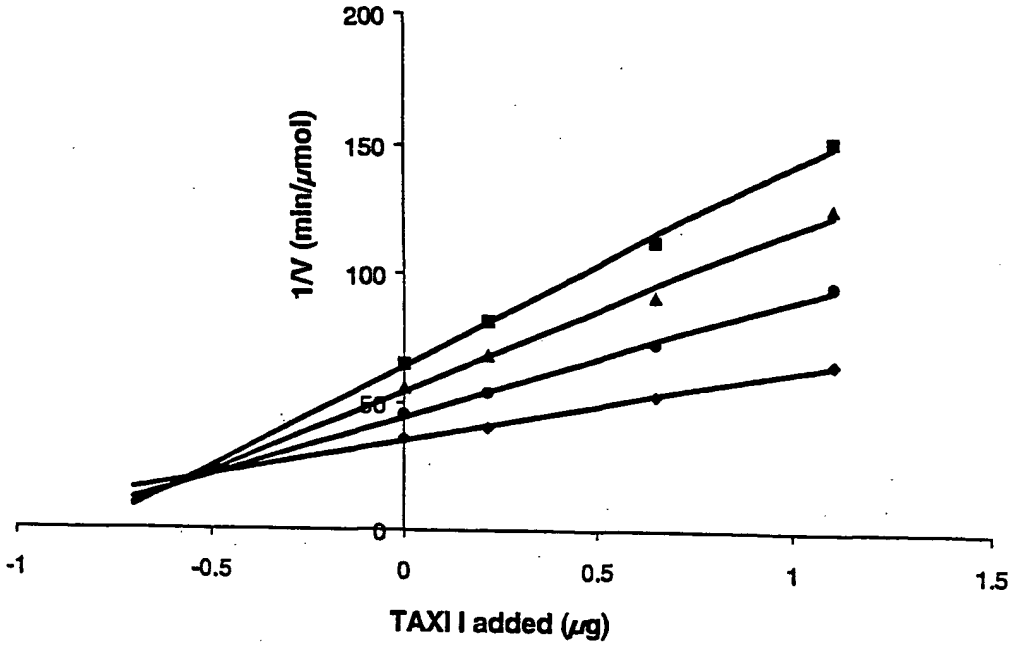

Figure 10

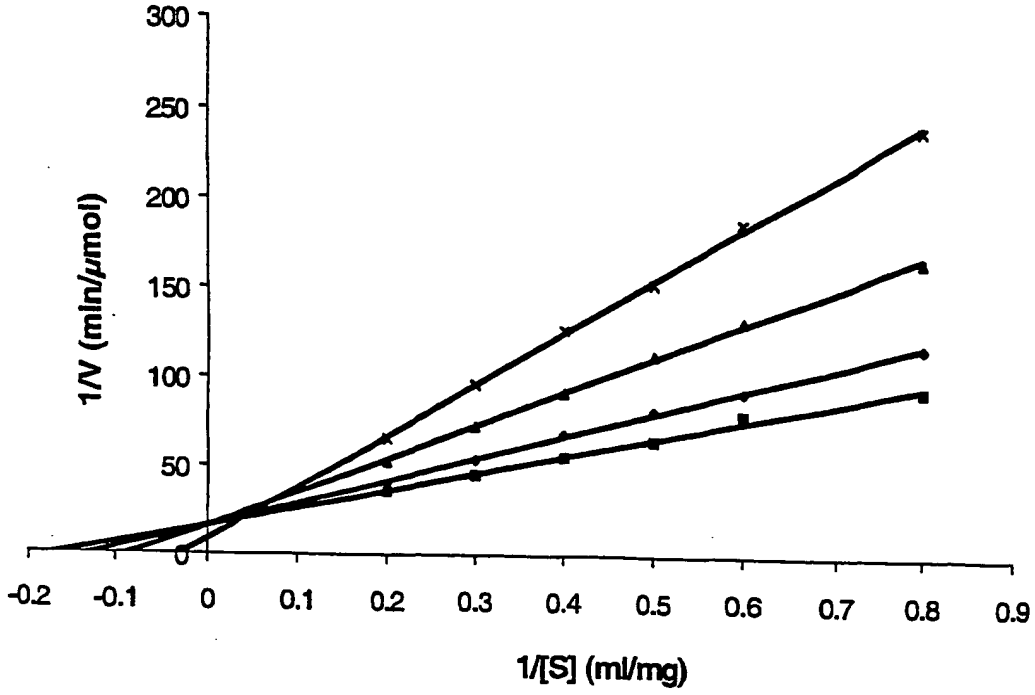
300
250
200
150
100
50
0
1/V (mln/µmol)
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/[S] (ml/mg)

Figure 11

A280
3
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
700
800
Elution volume (mL)
NaCl concentration (M)
0.6
0.5
0.4
0.3
0.2
0.1
0
Inhibition activity (%)
100
75
50
25
0

Figure 12

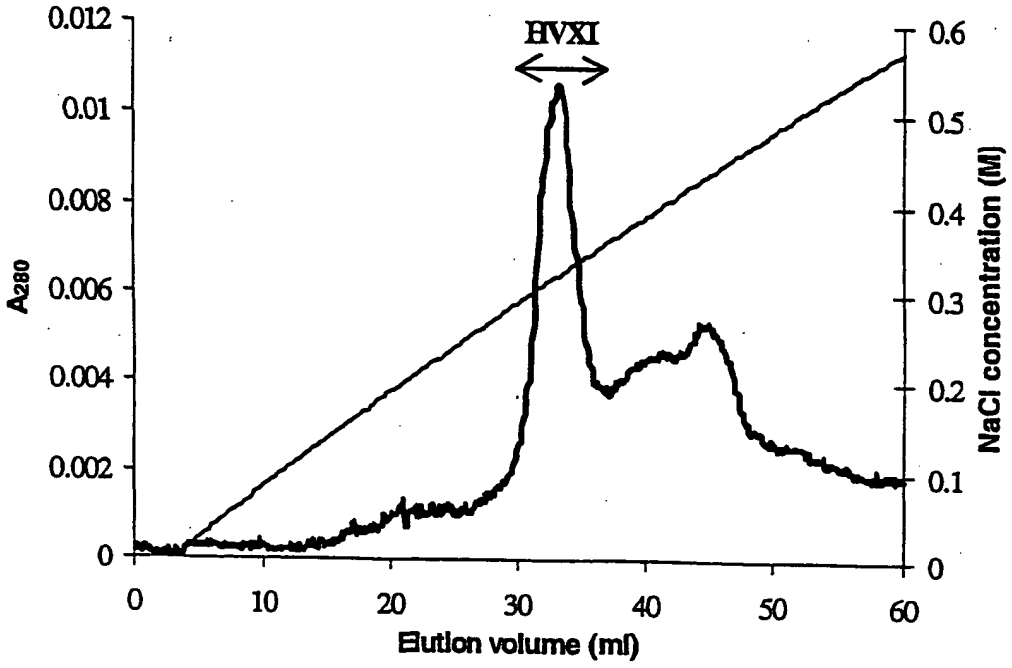
HVXI
0.012
0.01
0.008
0.006
0.004
0.002
0
A280
0.6
0.5
0.4
0.3
0.2
0.1
0
NaCl concentration (M)
0
10
20
30
40
50
60
Elution volume (ml)

Figure 13

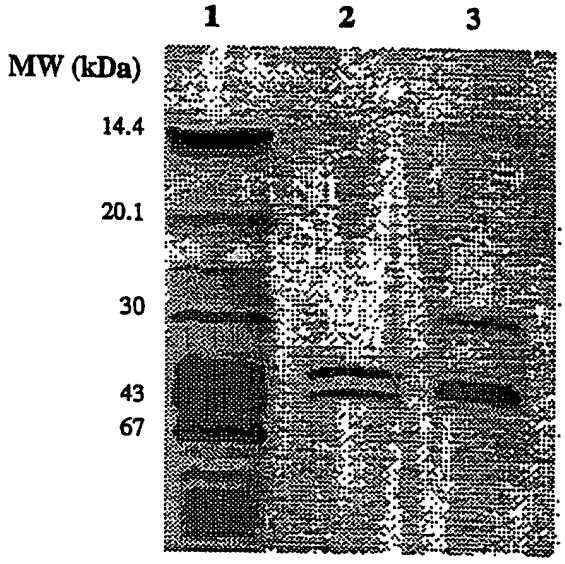

Figure 14

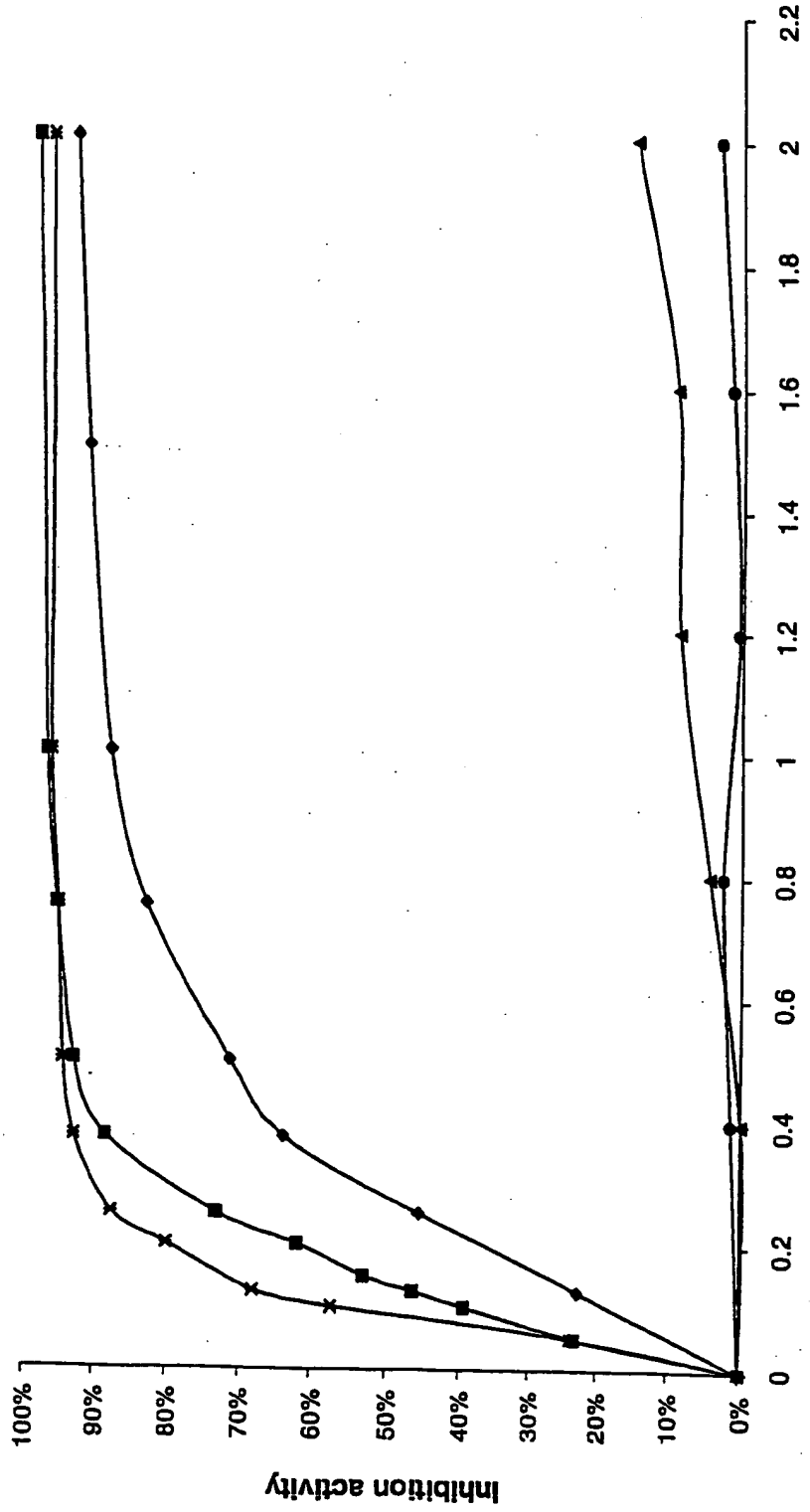
Inhibition activity
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
HVXI added (µg)

Figure 15

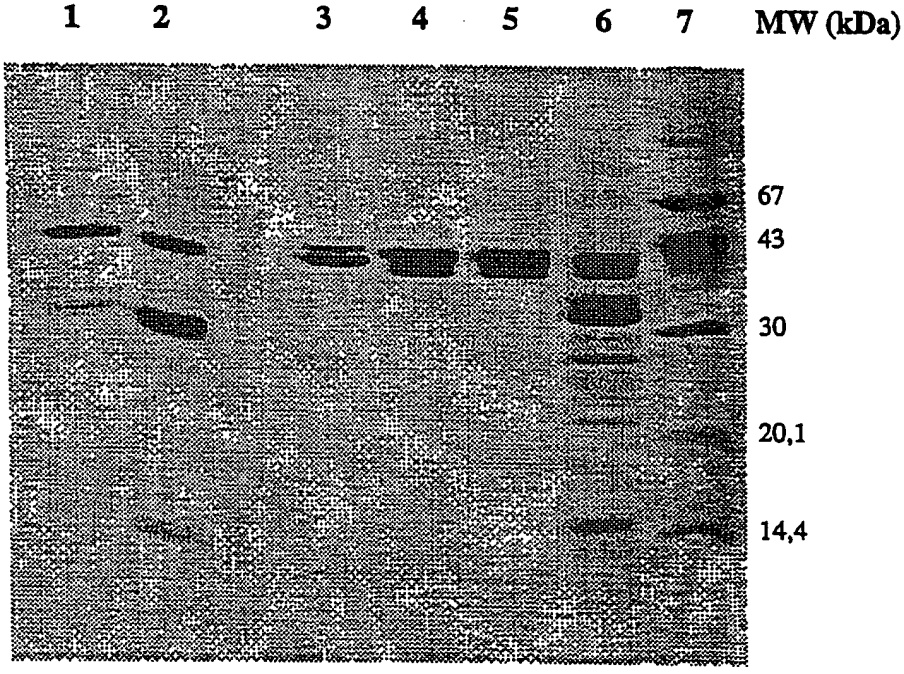

Figure 16

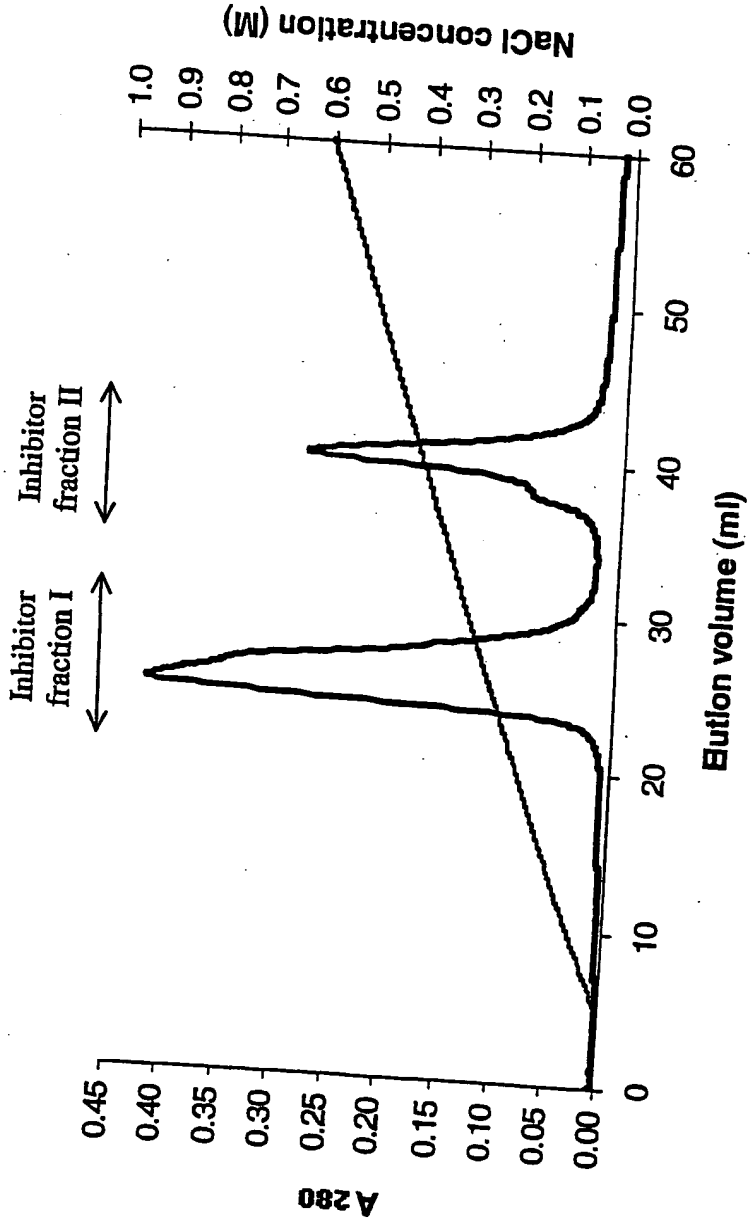
NaCl concentration (M)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A 280
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
Elution volume (ml)
Inhibitor fraction I
Inhibitor fraction II

Figure 17

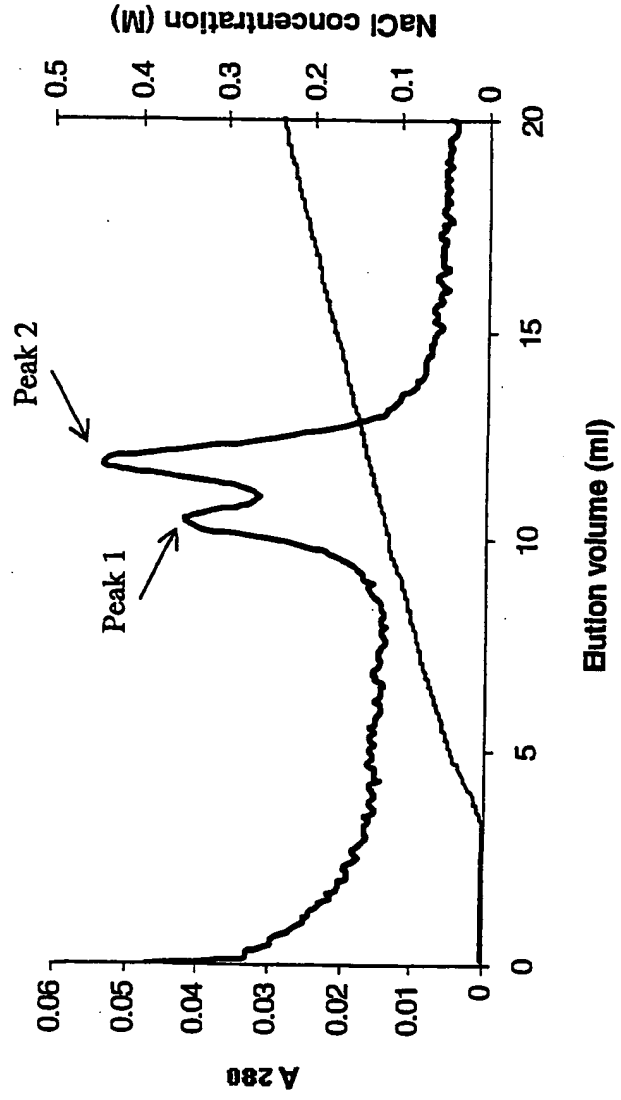
NaCl concentration (M)
0.5
0.4
0.3
0.2
0.1
0
A 280
0.06
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
Elution volume (ml)
Peak 2
Peak 1

Figure 18

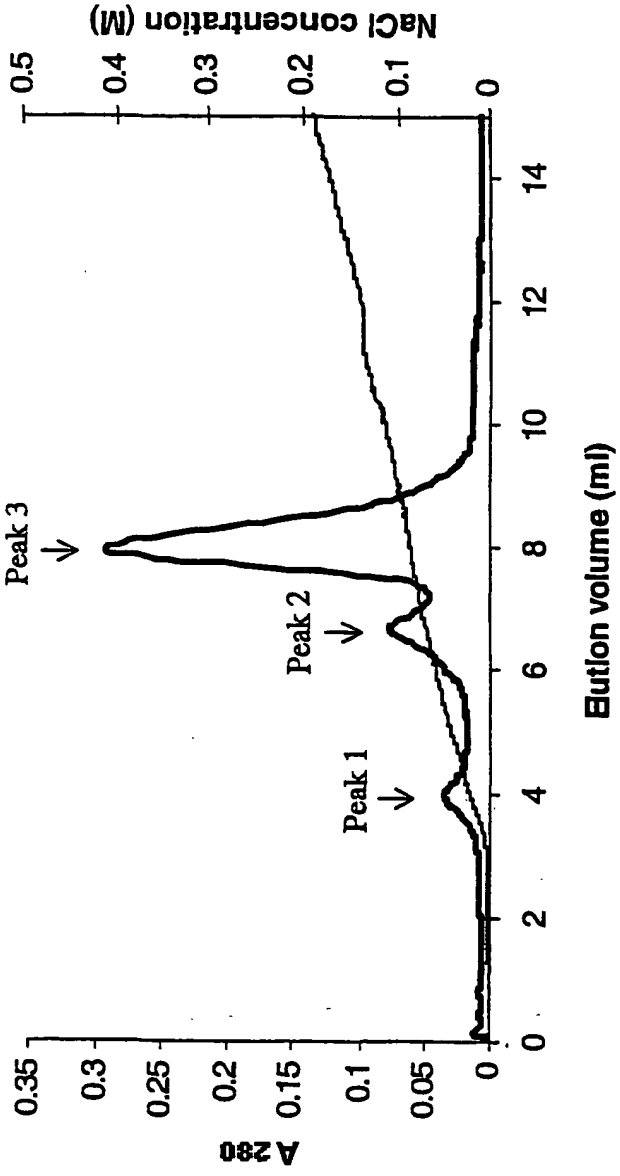
NaCl concentration (M)
0.5
0.4
0.3
0.2
0.1
0
A280
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
14
Elution volume (ml)
Peak 1
Peak 2
Peak 3

Figure 19

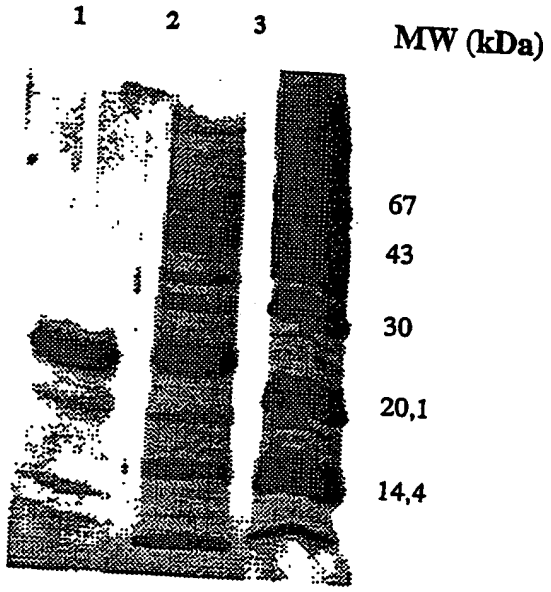

Figure 20

CCA	AGA	TCT	CTG	CCA	GTT	CTG	GCA	CCT	GTG	ACC	AAA	GAT	CCA
Pro	Arg	Ser	Leu	Pro	Val	Leu	Ala	Pro	Val	Thr	Lys	Asp	Pro

TAXI SEQ ID No.21

GCA	ACC	TCC	CTC	TAC	ACA	ATC	CCC	_____	CTG	CCG	CAC	TTT
Ala	Thr	Ser	Leu	Tyr	Thr	Ile	Pro		Leu	Pro	His	Phe

ACG	GGT	TGC	GGC	GGC	CTG	TAA	AGA	TCT	AGG
Thr	Gly	Cys	Gly	Gly	Leu	Stop	Arg	Ser	Arg

Figure 21

Thioredoxin — Enterokinase recognition site

AAC	CTG	GCC	GGC	TCT	GGA	TCC	GGT	GAT	GAC	GAT	GAC	AAG	CTC
Asn	Leu	Ala	Gly	Ser	Gly	Ser	Gly	Asp	Asp	Asp	Asp	Lys	Leu

PCR product

GCC	CTT	CCA	AGA	TCT	CTG	CCA_____	CTG	TAA	AGA	TCT	AGG	AAG
Ala	Leu	Pro	Arg	Ser	Leu	Pro	Leu	Stop	Arg	Ser	Arg	Lys

Figure 22

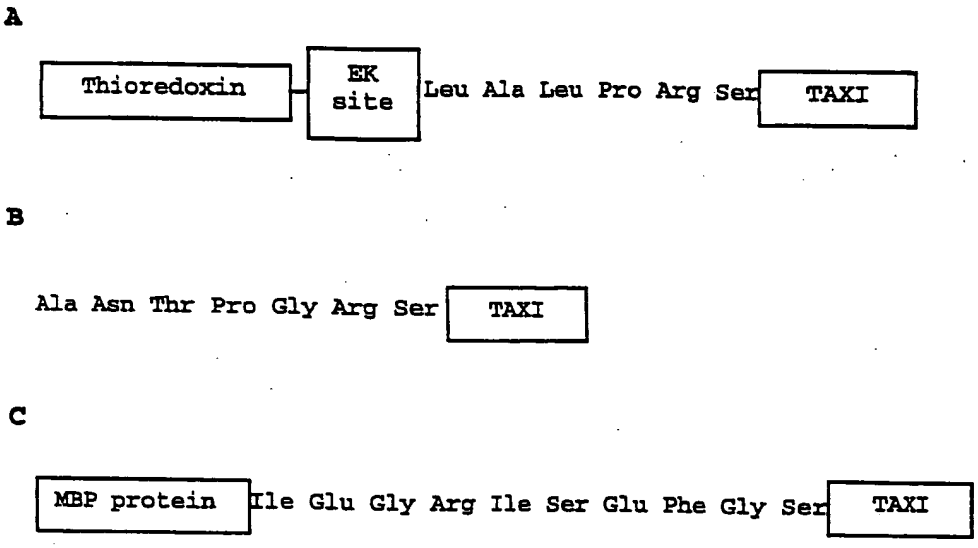
A
Thioredoxin
EK site
Leu Ala Leu Pro Arg Ser
TAXI
B
Ala Asn Thr Pro Gly Arg Ser
TAXI
C
MBP protein
Ile Glu Gly Arg Ile Ser Glu Phe Gly Ser
TAXI

Figure 23

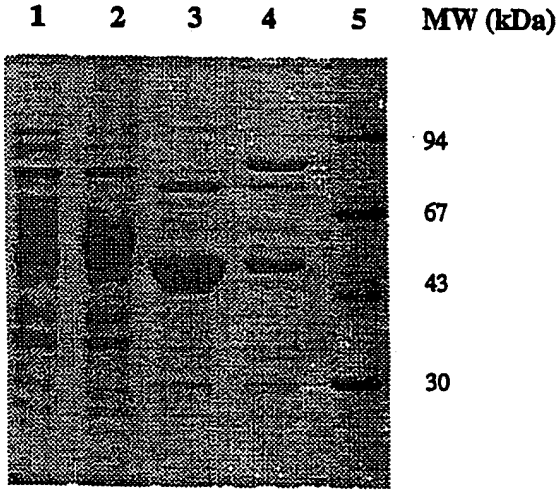

Figure 24

PelB

TTA TTA CTC GCG GCC CAG CCG GCC ATG GCT GCC AAC ACA CCC
Leu Leu Leu Ala Ala Gln Pro Ala Met Ala Ala Asn Thr Pro

PCR product

GGG AGA TCT CTG CCA _____ GGC CTG TAA AGA TCT TTC GAA
Gly Arg Ser Leu Pro Gly Leu Stop Arg Ser Phe Glu

Figure 25

malE Factor Xa recognition site

AAC	AAT	AAC	AAC	AAC	CTC	GGG	ATC	GAG	GGA	AGG	ATT	TCA	GAA
Asn	Asn	Asn	Asn	Asn	Leu	Gly	Ile	Glu	Gly	Arg	Ile	Ser	Glu

PCR product

TTC	GGA	TCT	CTG	CCA		GGC	CTG	TAA	AGA	TCC	TCT	AGA
Phe	Gly	Ser	*Leu*	*Pro*		*Gly*	*Leu*	Stop	Arg	Ser	Ser	Arg

Figure 26

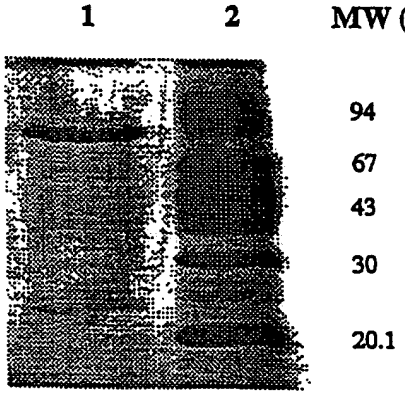

Figure 27

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/BE 01/00106

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/42 C07K14/415 C07K1/16 C07K16/16 A21D8/00 A23L1/105

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A21D A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
SEQUENCE SEARCH, MEDLINE, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEBYSER W ET AL: "TRITICUM ZESTIVUM XYLANASE INHIBITOR (TAXI), A NEW CLASS OF ENZYME INHIBITOR AFFECTING BREADMAKING PERFORMANCE" JOURNAL OF CEREAL SCIENCE, ACADEMIC PRESS LTD, XX, vol. 30, no. 1, July 1999 (1999-07), pages 39-43, XP000925298 ISSN: 0733-5210 cited in the application the whole document	1-161
X	WO 98'49278 A (DEBYSER WINOK ;DELCOUR JAN (BE); LEUVEN K U RES & DEV (BE)) 5 November 1998 (1998-11-05) cited in the application the whole document	1-161

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2001	07/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Herrmann, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int al Application No
PCT/BE 01/00106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCLAUCHLAN W R ET AL: "A NOVEL CLASS OF PROTEIN FROM WHEAT WHICH INHIBITS XYLANASES" BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 338, no. 2, 1 March 1999 (1999-03-01), pages 441-446, XP000925393 ISSN: 0264-6021 the whole document	1-161
X	EP 0 979 830 A (TNO) 16 February 2000 (2000-02-16) the whole document	1-161
P,X	WO 00 39289 A (SOERENSEN JENS FRISBAEK ;DANISCO (DK); SIBBESEN OLE (DK)) 6 July 2000 (2000-07-06) the whole document	1-161
P,X	GEBRUERS KURT ET AL: "Triticum aestivum L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities." BIOCHEMICAL JOURNAL, vol. 353, no. 2, 2001, pages 239-244, XP001022202 ISSN: 0264-6021 the whole document	1-161
T	GOESAERT H ET AL: "Purification and partial characterization of an endoxylanase inhibitor from barley." CEREAL CHEMISTRY, vol. 78, no. 4, July 2001 (2001-07), pages 453-457, XP001033832 ISSN: 0009-0352 the whole document	1-161

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-161 (all partially)

Support, disclosure, clarity:
Present set of claims relates to products and methods defined by reference to a desirable characteristic or property, namely inhibitors of cellulose, xylan, arabinoxylan or beta-glucan degrading enzymes. The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Art. 6 PCT and/or disclosure within the meaning of Art. 5 PCT for only inhibitors of "endoxylanases" (see p. 16, l. 3-8 of present description). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Art. 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Clarity, conciseness:
In view of the large number and also the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Art. 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

Clarity, conciseness:
Present set of claims relates to an extremely large number of possible endoxylanase inhibitors and methods for the isolation of such peptides. In fact, the claims contain so many options, variables, possible permutations and provisos that a lack of clarity and conciseness within the meaning of Art. 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Besides TAXI I, TAXI II and HvXI numerous other endoxylanse inhibitors and variants, homologues and fragments thereof are claimed.

CONSEQUENTLY, THE SEARCH HAS BEEN CARRIED OUT FOR THE GENERAL ASPECT OF THE INVENTION, NAMELY "PROTEINS WHICH INHIBIT ENDOXYLANASES".

Unity:
According to Art. 3(4)(iii) and Rule 13 PCT an application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept, i.e., having at least one common technical feature defining a contribution over the prior art.

The original common concept which might have linked the above mentioned inventions is the provision of endoxylanase inhibitors. This common concept is however not novel with regard to prior art: The documents mentioned in this search report all disclose proteins which inhibit endoxylanases.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Since no other technical feature can be distinguished which might link the subject-matter of present claims, each single endoxylanse inhibitor and each method for the separation or isolation of such inhibitors claimed represents an independent invention. Due to the reasons given above (lack of clarity and conciseness) it is however impossible to determine the exact number of individual endoxylanase inhibitors claimed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

'ormation on patent family members

Ir onal Application No
PCT/BE 01/00106

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9849278 A	05-11-1998	AU	7761198 A	24-11-1998
		BR	9809348 A	04-07-2000
		CN	1254374 T	24-05-2000
		WO	9849278 A1	05-11-1998
		EP	0996709 A1	03-05-2000
EP 0979830 A	16-02-2000	EP	0979830 A1	16-02-2000
WO 0039289 A	06-07-2000	AU	1676600 A	31-07-2000
		BR	9916507 A	02-10-2001
		EP	1141254 A1	10-10-2001
		FR	2788781 A1	28-07-2000
		WO	0039289 A2	06-07-2000

Evidence Appendix (b)

List of 500 of 892 U.S. Patents which include words BESTFIT and protein

Debyser et al
Serial No. 09/403,625
Evidence Appendix (b)

1	6,506,962	Acquired resistance genes in plants
2	6,506,603	Shuffling polynucleotides by incomplete extension
3	6,506,602	Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
4	6,506,569	Antibodies to human tumor necrosis factor receptor TR10
5	6,506,565	Plant regulatory sequences for selective control of gene expression
6	6,506,559	Genetic inhibition by double-stranded RNA
7	6,504,084	Maize NPR1 polynucleotides and methods of use
8	6,504,083	Maize Gos-2 promoters
9	6,504,082	Ecdysone receptors and methods for their use
10	6,504,010	Compositions and methods for the therapy and diagnosis of lung cancer
11	6,504,009	Transcriptional regulator
12	6,503,744	Campylobacter glycosyltransferases for biosynthesis of gangliosides and ganglioside mimics
13	6,503,735	Polynucleotides encoding chemokine .beta.-15
14	6,503,729	Selected polynucleotide and polypeptide sequences of the methanogenic archaeon, methanococcus jannashii
15	6,503,703	Identification and use of antiviral compounds that inhibit interaction of host cell proteins and viral proteins required for viral replication
16	6,503,184	Human tumor necrosis factor receptor-like proteins TR11, TR11SV1 and TR11SV2
17	6,501,008	Maize endo-1,3;1,4-.beta.-glucanase nucleic acid
18	6,501,006	Nucleic acids conferring chilling tolerance
19	6,500,667	Aspartyl protease 2 (Asp2) antisense oligonucleotides
20	6,500,663	Unique associated Kaposi's sarcoma virus sequences and uses thereof
21	6,500,661	Enzymatic conversion of GDP-mannose to GDP-fucose
22	6,500,643	Human high affinity choline transporter
23	6,500,639	DNA shuffling to produce nucleic acids for mycotoxin detoxification
24	6,500,626	Cell death regulators
25	6,500,617	Optimization of pest resistance genes using DNA shuffling
26	6,500,613	Pneumococcal surface proteins and uses thereof
27	6,500,431	Inhibitors of angiogenesis and tumor growth
28	6,500,211	Mutant EGIII cellulase, DNA encoding such EGIII compositions and methods for obtaining same
29	6,495,667	Il-b30 antibodies

30	6,495,664	Fluorescent protein sensors of post-translational modifications
31	6,495,520	Apoptosis Inducing Molecule II and methods of use
32	6,495,348	Mitomycin biosynthetic gene cluster
33	6,495,322	RAIDD, a novel death adaptor molecule
34	6,495,129	Methods of inhibiting hematopoietic stem cells using human myeloid progenitor inhibitory factor-1 (MPIF-1) (Ckbeta-8/MIP-3)
35	6,495,128	Human chemokine .beta.-7 deletion and substitution proteins
36	6,492,577	Leafy cotyledon2 genes and their uses
37	6,492,158	Human kinesin protein HsKif6
38	6,492,151	Motor proteins and methods for their use
39	6,489,453	Chandra: a TH1-specific gene
40	6,489,146	End-complementary polymerase reaction
41	6,489,145	Method of DNA shuffling
42	6,489,138	Human ependymin
43	6,488,925	Macrophage inflammatory protein-4 (MIP-4) polypeptides
44	6,486,302	Hm2 cDNA and related polypeptide
45	6,486,301	Interleukin-20
46	6,485,925	Anthrax lethal factor is a MAPK kinase protease
47	6,485,719	Methods for inhibiting angiogenesis with leukocyte adhesion inhibitor-1 (LAI-1) polypeptides
48	6,484,105	Method for obtaining a plant with a genetic lesion in a gene sequence
49	6,483,011	Modified ADP-glucose pyrophosphorylase for improvement and optimization of plant phenotypes
50	6,482,923	Interleukin 17-like receptor protein
51	6,482,799	Self-preserving multipurpose ophthalmic solutions incorporating a polypeptide antimicrobial
52	6,482,647	Evolving susceptibility of cellular receptors to viral infection by recursive recombination
53	6,482,621	Compositions and methods for fumonisin detoxification
54	6,482,600	Breast cancer associated nucleic acid sequences and their associated proteins
55	6,479,731	Pi-ta gene conferring fungal disease resistance to plants
56	6,479,730	DNA Ligase II orthologue uses thereof
57	6,479,652	Oligonucleotide mediated nucleic acid recombination
58	6,479,642	Cortistatin: neuropeptides
59	6,479,634	IL-B30 proteins
60	6,479,629	Maize histone deacetylases and their use
61	6,479,258	Non-stochastic generation of genetic vaccines

62	6,479,254	Apoptosis inducing molecule II
63	6,476,297	Meiosis promoter
64	6,476,296	Nucleic acids that control seed and fruit development in plants
65	6,475,793	Genomic sequence of Rhizobium sp. NGR 234 symbiotic plasmid
66	6,475,789	Human telomerase catalytic subunit: diagnostic and therapeutic methods
67	6,475,784	Inhibition of angiogenesis by delivery of nucleic acids encoding anti-angiogenic polypeptides
68	6,475,762	Phytase enzymes nucleic acids encoding phytase enzymes and vectors and host cells incorporating same
69	6,475,734	Polyhydroxyalkanoate synthase genes
70	6,475,492	Peptides and assays for the diagnosis of lyme disease
71	6,472,512	Keratinocyte derived interferon
72	6,472,197	GRB2 associating polypeptides and nucleic acids encoding therefor
73	6,472,140	alpha.-2- macroglobulin therapies and drug screening methods for Alzheimer's disease.
74	6,470,277	Techniques for facilitating identification of candidate genes
75	6,469,230	Starch debranching enzymes
76	6,468,984	DNA vaccine for protecting an avian against infectious bursal disease virus
77	6,468,978	Active hedgehog protein conjugate
78	6,468,768	Galectin 9 and 10SV polynucleotides
79	6,465,716	Nod factor binding protein from legume roots
80	6,465,633	Compositions and methods of their use in the treatment, prevention and diagnosis of tuberculosis
81	6,465,238	Gene encoding phosphoglucoisomerase
82	6,465,212	Leukotriene receptor
83	6,465,203	Glucan-containing compositions and paper
84	6,465,181	Reagents and methods useful for detecting diseases of the prostate
85	6,462,258	Plant expression constructs
86	6,462,254	Dual-tagged proteins and their uses
87	6,461,863	Modifying insect cell gylcosylation pathways with baculovirus expression vectors
88	6,461,855	Motor proteins and methods for their use
89	6,461,836	Molecular cloning of a 7TM receptor (AxOR34) and screening methods thereof
90	6,461,823	Death domain containing receptor-4 antibodies
91	6,458,930	Aspergillus fumigatus cofilin

92	6,458,532	Polynucleotides encoding IMP.18p myo-inositol monophosphatase and methods of detecting said polynucleotides
93	6,458,530	Selecting tag nucleic acids
94	6,455,668	Methods of diagnosing colorectal cancer, compositions, and methods of screening for colorectal cancer modulators
95	6,455,297	Methods and compositions for regulating cell death and enhancing disease resistance to plant pathogens
96	6,455,293	Human kinesin-like protein HSKIF21B
97	6,455,254	Sequence based screening
98	6,455,040	Tumor necrosis factor receptor
99	6,452,069	SF3 promoter and methods of use
100	6,451,759	Noncleavable Fas ligand
101	6,451,562	Polypeptides encoding myeloid progenitor inhibitory factor-1 (MPIF-1) polynucleotides
102	6,451,539	Expression vectors, transfection systems, and method of use thereof
103	6,448,234	Compounds and methods for treatment and diagnosis of chlamydial infection
104	6,448,035	Family of immunoregulators designated leukocyte immunoglobulin-like receptor (LIR)
105	6,448,026	Screening assays for modulators of human kinesin protein HsKrp5
106	6,448,025	Motor proteins and methods for their use
107	6,448,020	Molecules associated with the human suppressor of fused gene
108	6,444,874	Hydroperoxide lyase gene from maize and methods of use
109	6,444,791	Methods and compositions for the treatment of keratoconus using protease inhibitors
110	6,444,790	Peptidoglycan recognition proteins
111	6,444,468	Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
112	6,444,440	Vanilloid receptor-2
113	6,441,151	Plant prohibition genes and their use
114	6,441,134	Isolated Candida albicans oligopeptide transporter gene
115	6,440,934	Angiogenically effective unit dose of FGF-2 and method of use
116	6,440,731	Polynucleotides encoding HsKrp5 a kinesin related protein
117	6,440,726	Expression vectors comprising multiple shear stress responsive elements (SSRE) and methods of use for

		treating disorders related to vasculogenesis and/or angiogenesis in a shear stress environment
118	6,440,698	Alzheimer's disease secretase, APP substrates therefor, and uses therefor
119	6,440,694	TNF-related death ligand
120	6,440,686	Methods for screening and therapeutic applications of kinesin modulators
121	6,440,685	Screening assays for modulators of human kinesin protein HsKif16b
122	6,440,684	Methods of identifying modulators of kinesin motor proteins
123	6,440,677	Nucleic acid affinity columns
124	6,440,668	Method of DNA shuffling with polynucleotides produced by blocking or interrupting a synthesis or amplification process
125	6,437,115	Nucleic acids encoding KSP, a kinesin motor protein
126	6,436,686	Motor proteins and methods for their use
127	6,436,675	Use of codon-varied oligonucleotide synthesis for synthetic shuffling
128	6,433,249	Use of .beta.-glucosidase to enhance disease resistance and resistance to insects in crop plants
129	6,433,147	Death domain containing receptor-4
130	6,433,145	Keratinocyte derived interferon
131	6,432,916	Compounds and methods for treatment and diagnosis of chlamydial infection
132	6,432,707	Compositions and methods for the therapy and diagnosis of breast cancer
133	6,432,678	Macaca cynomolgus IL 18
134	6,432,671	Tryparedoxin, expression plasmid, process of production, method of use, test kit, and pharmaceutical composition
135	6,432,666	Dendritic cell receptor
136	6,432,660	Motor proteins and methods for their use
137	6,432,659	Motor proteins and methods for their use
138	6,432,645	Beta subunits of Slo family potassium channels
139	6,432,628	Caspase-14, an apoptotic protease, nucleic acids encoding and methods of use
140	6,429,362	Maize PR-1 gene promoters
141	6,429,304	Nucleic acids encoding a katanin p60 subunit
142	6,429,293	Sculpin-type antifreeze polypeptides and nucleic acids
143	6,429,005	Motor proteins and methods for their use
144	6,428,980	Nucleic acids encoding RIP3 associated cell cycle proteins
145	6,428,788	Compositions and methods for specifically targeting tumors
146	6,426,224	Oligonucleotide mediated nucleic acid recombination
147	6,426,207	Motor proteins and methods for their use

148 6,426,197 Polynucleotides encoding a human potassium channel
149 6,426,193 Screening assays for modulators of human kinesin protein HsKif21b
150 6,426,075 Protease-activatable pseudomonas exotoxin A-like proproteins
151 6,426,072 Compositions and methods for the therapy and diagnosis of lung cancer
152 6,423,544 Compositions and methods for producing recombinant virions
153 6,423,542 Oligonucleotide mediated nucleic acid recombination
154 6,423,513 Polynucleotides encoding protease-activatable pseudomonas exotoxin a-like proproteins
155 6,421,613 Data processing of the maize prolifera genetic sequence
156 6,420,544 Polynucleotide and polypeptide sequences encoding murine organic anion transporter 5 (mOATP5)
157 6,420,534 Alzheimer's disease secretase, APP substrates therefor, and uses thereof
158 6,420,175 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
159 6,420,162 Nucleic acids encoding hskif16a, a kinesin motor protein
160 6,420,137 Nucleic acid encoding human neurotensin subtype 2 receptor
161 6,420,118 Peptides and peptidomimetics with structural similarity to human p53 that activate p53 function
162 6,420,116 Antimicrobial peptide
163 6,416,973 Nucleic acids encoding mammalian cell membrane protein MDL-1
164 6,416,966 Screening assays for modulators of human kinesin protein HsKif6
165 6,414,221 Transiently activated stress-inducible plant promoters
166 6,414,121 Human kinesin protein KSP
167 6,413,774 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
168 6,413,751 DNA adenine methyltransferases and uses thereof
169 6,413,745 Recombination of insertion modified nucleic acids
170 6,413,741 Human elk a voltage-gated potassium channel subunit
171 6,410,828 Regulatory sequences useful for gene expression in plant embryo tissue
172 6,410,687 Polypeptides for the detection of microtubule depolymerization inhibitors
173 6,410,245 Compositions and methods for detecting ligand-dependent nuclear receptor and coactivator interactions
174 6,410,233 Isolation and identification of control sequences and genes modulated by transcription factors

175 6,410,023 Recombinant parainfluenza virus vaccines attenuated by deletion or ablation of a non-essential gene
176 6,409,648 Polynucleotides encoding TRF1 binding proteins
177 6,407,315 Seed-preferred promoter from barley
178 6,407,211 Chimeric natriuretic peptides
179 6,407,046 Mutant EGIII cellulase, DNA encoding such EGIII compositions and methods for obtaining same
180 6,406,910 Recombination of insertion modified nucleic acids
181 6,406,907 Bovine tumor necrosis factor receptor-1 and methods of use
182 6,406,867 Antibody to human endokine alpha and methods of use
183 6,403,862 Seed-preferred promoter from maize
184 6,403,860 Ku80 homologue and uses thereof
185 6,403,770 Antibodies to neutrokine-alpha
186 6,403,768 Manipulation of Mlo genes to enhance disease resistance in plants
187 6,403,557 Fibroblast growth factor-13
188 6,403,372 Aspergillus fumigatus profilin
189 6,403,351 Pyruvate carboxylase polypeptide from Corynebacterium glutamicum
190 6,399,573 Interleukin-1 receptor antagonist beta (IL-1rabeta)
191 6,399,346 Human kinesin-like protein HsKif16b
192 6,399,294 Nucleotide sequences of HIV-1 type (or subtype) O retrovirus antigens
193 6,395,962 Enhancing expression of a silenced target sequence in plants using plant viral enhancers and amplicons
194 6,395,547 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
195 6,395,540 Nucleic acids encoding HsKifC2, a kinesin motor protein
196 6,395,527 Motor proteins and methods for their use
197 6,395,514 Polynucleotides encoding chemokine.alpha.-5
198 6,395,306 Bee venom protein and gene encoding same
199 6,392,126 Adenosine deaminase homologues and uses thereof
200 6,392,026 Binding domains from plasmodium vivax and plasmodium falciparum erythrocyte binding proteins
201 6,392,024 Tenebrio antifreeze proteins
202 6,392,015 Method of identifying modulators of HIV-1 Vpu and Gag interaction with U binding protein (Ubp)
203 6,391,613 Motor proteins and methods for their use
204 6,391,601 Motor proteins and methods for their use
205 6,391,584 Expression-cloning method for identifying target proteins for eukaryotic tyrosine kinases and novel target proteins
206 6,391,573 Screening assays for modulators of human kinesin protein HSKIP3B
207 6,391,564 Methods and compositions utilizing Rad51

208 6,391,552 Enhancing transfection efficiency of vectors by recursive recombination
209 6,391,316 Vaccine compositions comprising Haemophilus somnus transferrin-binding proteins and methods of use
210 6,388,171 Compositions and methods for fumonisin detoxification
211 6,388,169 Maize orthologues of bacterial RecA proteins
212 6,388,066 MAR/SAR elements flanking RSYN7-driven construct
213 6,388,062 Modified p53 tetramerization domains having hydrophobic amino acid substitutions
214 6,388,052 NF-AT polypeptides and polynucleotides
215 6,387,702 Enhancing cell competence by recursive sequence recombination
216 6,387,679 Motor proteins and method for their use
217 6,387,658 PCNA-associated cell cycle proteins, compositions and methods of use
218 6,387,644 Motor proteins and methods for their use
219 6,387,641 Crystallized P38 complexes
220 6,384,302 Trypsin inhibitors with insecticidal properties obtained from Pentaclethra macroloba
221 6,384,203 Family of immunoregulators designated leukocyte immunoglobulin-like receptors (LIR)
222 6,383,796 Nucleic acids encoding HSKIF21B, a kinesin motor protein
223 6,383,778 Nucleic acids encoding a G-protein coupled receptor involved in sensory transduction
224 6,380,183 Treatment of diseases involving cyst formation
225 6,379,964 Evolution of whole cells and organisms by recursive sequence recombination
226 6,379,941 Human kinesin-related protein HsKrp5
227 6,379,926 Polynucleotides encoding chemokine .beta.-6 antagonists
228 6,379,925 Angiogenic modulation by notch signal transduction
229 6,379,923 ELL2, a new member of an ELL family of RNA polymerase II elongation factors
230 6,379,912 Motor proteins and method for their use
231 6,379,671 Reagents and methods useful for detecting diseases of the breast
232 6,376,751 Nucleic acids encoding EMF1 that control reproductive development in plants
233 6,376,475 Control of immune responses by modulating activity of glycosyltransferases
234 6,376,246 Oligonucleotide mediated nucleic acid recombination
235 6,376,196 Recombinant neospora antigens and their uses
236 6,375,954 Size-variable strain-specific protective antigen for potomac horse fever
237 6,372,898 Human JAK3 variants

238 6,372,711 Methods for assaying human FSH using human FSH receptor
239 6,372,497 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
240 6,372,473 Tissue plasminogen activator-like protease
241 6,372,456 Polynucleotides encoding chemokine .alpha.-6
242 6,372,454 Nucleic acid molecules encoding Follistatin-3
243 6,372,215 Monoclonal antibodies to human CD6
244 6,369,296 Recombinant plant viral vectors
245 6,369,201 Myostatin multimers
246 6,368,861 Oligonucleotide mediated nucleic acid recombination
247 6,368,841 Human kinesin-related protein HsKip3b
248 6,368,826 IGF-1 receptor interacting proteins
249 6,368,823 Kv potassium channel polypeptides and polynucleotides
250 6,365,712 Methods and compositions for inhibiting inflammation and angiogenesis comprising a mammalian CD97 .alpha. subunit
251 6,365,408 Methods of evolving a polynucleotides by mutagenesis and recombination
252 6,365,377 Recombination of insertion modified nucleic acids
253 6,361,993 Human HSET motor proteins and methods for their use
254 6,361,975 Mouse aspartic secretase-2(mASP-2)
255 6,361,974 Exonuclease-mediated nucleic acid reassembly in directed evolution
256 6,358,742 Evolving conjugative transfer of DNA by recursive recombination
257 6,358,740 Recombination of insertion modified nucleic acids
258 6,358,725 Mouse aspartic secretase-1 (mASP1)
259 6,358,709 End selection in directed evolution
260 6,358,508 Antibodies to human tumor necrosis factor receptor TR9
261 6,355,471 Nucleic acids encoding Hskif16b, a kinesin motor protein
262 6,355,466 Motor proteins and methods for their use
263 6,355,465 Compounds
264 6,355,452 Human histamine H3 gene variant-2
265 6,355,447 Motor proteins and methods for their use
266 6,355,411 Virulence-associated nucleic acid sequences and uses thereof
267 6,352,859 Evolution of whole cells and organisms by recursive sequence recombination
268 6,352,842 Exonucease-mediated gene assembly in directed evolution
269 6,352,830 NF-AT polypeptides and polynucleotides and screening methods for immunosuppressive agents
270 6,352,694 Methods for inducing a population of T cells to proliferate using agents which recognize TCR/CD3 and ligands which stimulate an accessory molecule on the surface of the T cells

271 6,350,933 RG polynucleotides for conferring powdery mildew resistance in plants
272 6,350,597 Riboflavin synthase genes and enzymes and methods of use
273 6,348,586 Unique associated Kaposi's sarcoma virus sequences and uses thereof
274 6,348,573 Compositions and methods for identifying apoptosis signaling pathway inhibitors and activators
275 6,348,569 Spruce budworm antifreeze proteins, genes and method of using same
276 6,348,348 Human hairless gene and protein
277 6,346,655 Trichothecne-Resistant transgenic plants
278 6,346,410 Motor proteins and methods for their use
279 6,346,379 Thermostable DNA polymerases incorporating nucleoside triphosphates labeled with fluorescein family dyes
280 6,344,549 ATR-2 cell cycle checkpoint
281 6,344,356 Methods for recombining nucleic acids
282 6,344,316 Nucleic acid analysis techniques
283 RE37,543 DNA sequence useful for the production of polyhydroxyalkanoates
284 6,342,656 Regulation of source-sink relationships and responses to stress conditions in plants
285 6,342,495 Agonists and antagonists of peripheral-type benzodiazepine receptors
286 6,342,382 Glycosyltransferases for biosynthesis of oligosaccharides, and genes encoding them
287 6,342,379 Detection of transmembrane potentials by optical methods
288 6,342,363 Death domain containing receptor 4 nucleic acids and methods
289 6,340,743 Antibodies to PIGR stalk
290 6,339,144 Proteins having insecticidal activities and method of use
291 6,335,198 Evolution of whole cells and organisms by recursive sequence recombination
292 6,335,189 Motor proteins and methods for their use
293 6,335,016 Chicken embryo lethal orphan (CELO) virus
294 6,333,184 Motor proteins and methods for their use
295 6,333,172 Genes and proteins controlling cholesterol synthesis
296 6,333,168 Cloning, expression and uses of dorsalin-1
297 6,333,155 Exploiting genomics in the search for new drugs
298 6,331,430 Motor proteins and methods for their use
299 6,331,424 Motor proteins and methods for their use
300 6,331,412 Methods and compounds for modulating male fertility
301 6,329,568 Tospovirus resistance in plants
302 6,329,567 Methods for improving seeds

303 6,329,504 Antifungal polypeptide and methods for controlling plant pathogenic fungi
304 6,326,472 Human receptor proteins; related reagents and methods
305 6,326,466 Double-stranded RNA dependent protein kinase derived peptides to promote proliferation of cells and tissues in a controlled manner
306 6,326,204 Evolution of whole cells and organisms by recursive sequence recombination
307 6,323,030 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
308 6,323,019 Design of novel highly efficient HIV based packaging systems for gene therapy
309 6,320,102 Leafy cotyledon1 genes and their uses
310 6,319,714 Oligonucleotide mediated nucleic acid recombination
311 6,316,609 Nucleotide sequence of Escherichia coli pathogenicity islands
312 6,316,407 Antifungal polypeptide from alfalfa and methods for controlling plant pathogenic fungi
313 6,316,272 Methods of diagnosis of colorectal cancer and methods of screening for colorectal cancer modulators
314 6,316,253 Expression vectors, transfection systems, and method of use thereof
315 6,316,239 HKABY60 kinase family polypeptides
316 6,316,225 Human Prt1-like subunit protein (hPrt1) polynucleotides
317 6,316,195 Method for differentiating between the casual agents of karnal bunt wheat fungus and ryegrass smut using PCR
318 6,313,376 Maize aquaporins and uses thereof
319 6,313,375 Maize aquaporins and uses thereof
320 6,313,374 Method of using pelarogonium sp. as hyperaccumulators for remediating contaminated soil
321 6,312,941 Compositions and methods for identifying signaling pathway agonists and antagonists
322 6,312,937 Metalloproteinases
323 6,312,907 DbpA compositions and methods of use
324 6,312,899 NF-AT polypeptides and polynucleotides
325 6,310,273 Inhibiting apoptosis in plants using a baculovirus p35 protease inhibitor gene
326 6,309,858 T-type calcium channel variants; compositions thereof; and uses
327 6,309,822 Method for comparing copy number of nucleic acid sequences
328 6,309,634 Methods of treating Parkinson's disease using recombinant adeno-associated vector (rAAV)
329 6,307,020 Intracellular antifreeze polypeptides and nucleic acids
330 6,303,768 Methuselah gene, compositions and methods of use
331 6,303,749 Agouti and agouti-related peptide analogs

332	6,303,370	Tissue-specific regulatory elements
333	6,303,338	Pancreas-derived plasminogen activator inhibitor
334	6,303,301	Expression monitoring for gene function identification
335	6,303,295	Selenoproteins, coding sequences and methods
336	6,302,685	Human lysosomal protein and methods of its use
337	6,300,485	Myosin IXa and cyclic nucleotide gated channel-15 (CNGC-15) polynucleotides, polypeptides, compositions, methods, and uses thereof
338	6,300,477	Antibodies to human cystatin E
339	6,300,110	Peptides related to TPC2 and TPC3, two proteins that are coexpressed with telomerase activity
340	6,300,098	Human signal transduction serine/threonine kinase
341	6,297,053	Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
342	6,297,021	Ligand screening and design by X-ray crystallography
343	6,296,848	GRB2 associating polypeptides and nucleic acids encoding therefor
344	6,294,650	Inhibition of mammalian telomerase by peptide nucleic acids
345	6,294,379	Efficient AAV vectors
346	6,294,371	Motor proteins and methods for their use
347	6,294,343	Methods of diagnosing colorectal cancer, compositions, and methods of screening for colorectal cancer modulators
348	6,291,744	Nucleic acids encoding plant group 2 proteins and uses thereof
349	6,291,642	Mammalian cell cycle protein
350	6,291,242	Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
351	6,291,223	Mouse aspartic secretase-1 (mASP1)
352	6,291,220	Polynucleotides encoding phosphatidylinositol 3-kinases
353	6,291,205	Method of increasing production of disulfide bonded recombinant proteins by saccharomyces cerevisiae
354	6,288,303	Rice .beta.-glucanase enzymes and genes
355	6,287,862	Evolution of whole cells and organisms by recursive sequence recombination
356	6,287,861	Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
357	6,287,843	Maize histone deacetylases and their use
358	6,287,839	Cellulase producing actinomycetes, cellulase produced therefrom and method of producing same
359	6,287,575	Vaccine against papillomatous digital dermatitis (PDD)
360	6,284,949	Insect-resistant plants comprising a Bacillus thuringiensis gene
361	6,284,948	Genes and methods for control of nematodes in plants
362	6,284,486	Human oncogene induced secreted protein I

363 6,284,479 Substitutes for modified starch and latexes in paper manufacture
364 6,284,461 Use of inhibitors in reporter assays
365 6,284,456 Transcriptional coactivator that interacts with Tat protein and regulates its binding to TAR RNA, methods for modulating Tat transactivation, and uses therefor
366 6,280,950 Nucleic acid affinity columns
367 6,277,962 Receptor on the surface of activated t-cells: act-4
368 6,277,959 Isolated mammalian membrane protein genes; related reagents
369 6,277,638 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
370 6,274,380 Cacnglike3 polynucleotides and expression systems
371 6,274,353 Method and compositions for improved polynucleotide synthesis
372 6,271,439 Methods and compositions for regulating cell death and enhancing disease resistance to plant pathogens
373 6,271,437 Soybean gene promoters
374 6,271,014 Mammalian proteinases; related reagents and methods
375 6,270,956 Transcriptional coactivator that interacts with Tat protein and regulates its binding to TAR RNA, methods for modulating Tat transactivation, and uses therefor
376 6,268,198 Cellulases and coding sequences
377 6,268,189 Fungal lactate dehydrogenase gene and constructs for the expression thereof
378 6,267,956 Protein activator and apoptosis
379 6,265,637 Genetic control of flowering
380 6,265,636 Pyruvate dehydrogenase kinase polynucleotides, polypeptides and uses thereof
381 6,265,177 Enzyme assay for mutant firefly luciferase
382 6,265,158 Ataxia-telangiectasia gene and its genomic organization
383 6,262,334 Human genes and expression products: II
384 6,262,333 Human genes and gene expression products
385 6,262,233 Tissue factor pathway inhibitor-3
386 6,262,018 Hypersensitive response elicitor from Erwinia amylovora and its use
387 6,261,801 Nucleic acids encoding tumor necrosis factor receptor 5
388 6,261,769 Intergenic spacer target sequence for detecting and distinguishing Chlamydial species or strains
389 6,261,760 Regulation of the cell cycle by sterols
390 6,258,777 Human B-cell translocation genes-2 and 3
391 6,258,536 Expression monitoring of downstream genes in the BRCA1 pathway
392 6,255,055 c-myc coding region determinant-binding protein (CRD-BP) and its nucleic acid sequence

393 6,252,057 Protein targeting to glycogen
394 6,251,674 Evolution of whole cells and organisms by recursive sequence recombination
395 6,251,632 Canine factor VIII gene, protein and methods of use
396 6,248,876 Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases
397 6,248,582 Gene deleted recombinant FeLV proviral DNA for production of vaccines against FeLV
398 6,248,550 Assays for protein kinases using fluorescent protein substrates
399 6,248,543 Compositions and methods for screening antimicrobials
400 6,248,517 Decorin binding protein compositions and methods of use
401 6,245,886 Peptides and peptidomimetics with structural similarity to human P53 that activate P53 function
402 6,242,668 Strawberry endo-1,4-.beta.-glucanase genes and their uses
403 6,242,566 Ligand (ACT-4-L) to a receptor on the surface of activated CD4+ T-cells
404 6,242,238 Isolated nucleic acid molecule encoding mammalian endoglucuronidase and uses therefor
405 6,238,888 Keratinocyte growth factor-2 formulations
406 6,238,884 End selection in directed evolution
407 6,235,975 Leafy cotyledon1 genes and methods of modulating embryo development in transgenic plants
408 6,235,972 Maize Rad23 genes and uses thereof
409 6,235,883 Human monoclonal antibodies to epidermal growth factor receptor
410 6,235,881 Polypeptides encoded by novel HIV-2 proviruses
411 6,235,510 ppGaNTase-T6
412 6,235,278 Biological insect control agents expressing insect-specific toxin genes, methods and compositions
413 6,232,527 Maize Rad2/FEN-1 orthologues and uses thereof
414 6,232,457 Recombinant vanadium haloperoxidases and their uses
415 6,232,110 Coding sequence for protein phosphatase methylesterase, recombinant DNA molecules and methods
416 6,232,100 Cortistatin Polypeptides
417 6,229,064 Nucleic acids that control endosperm development in plants
418 6,228,992 Proteins for control of nematodes in plants
419 6,228,623 Polyhydroxyalkanoates of narrow molecular weight distribution prepared in transgenic plants
420 6,228,591 Polycystic kidney disease PKD2 gene and uses thereof
421 6,225,532 Tomato CF-5 gene encoding a disease resistance polypeptide
422 6,225,456 Ras suppressor SUR-5

423 6,225,086 Polynucleotides encoding ankyrin proteins
424 6,222,095 Sequences from auxin-induced gene products targeting fusion proteins for degradation
425 6,222,019 Human IRAK-2 antibodies
426 6,221,597 Essential genes of yeast as targets for antifungal agents, herbicides, insecticides and anti-proliferative drugs
427 6,218,523 Prostate cancer-specific marker
428 6,215,048 Nucleic acid sequences encoding an antifungal polypeptide, aly AFP from alyssum and methods for their use
429 6,214,588 Factors which modify gene transcription and methods of use therefor
430 6,214,580 Human tumor necrosis factor receptor tr10
431 6,214,355 DbpA compositions
432 6,211,440 Hm2 cDNA from maize encoding disease resistance polypeptide
433 6,211,435 Amino polyol amine oxidase polynucleotides and related polypeptides and methods of use
434 6,211,434 Amino polyol amine oxidase polynucleotides and related polypeptides and methods of use
435 6,211,433 Manipulation of Mlo genes to enhance disease resistance in plants
436 6,211,430 FbLate promoter
437 6,211,336 Ataxia-telangiectasia gene
438 6,211,164 Antisense oligonucleotides of the human chk1 gene and uses thereof
439 6,210,933 Recombinant .alpha.-2,3-sialyltransferases and their uses
440 6,210,671 Humanized antibodies reactive with L-selectin
441 6,210,670 Cross-reacting monoclonal antibodies specific for E-selectin and P-selectin
442 6,207,432 Tyrosylprotein sulfotransferases and methods of use thereof
443 6,207,414 Tyrosylprotein sulfotransferases and methods of use thereof
444 6,207,380 Reagents and methods useful for detecting diseases of the urinary tract
445 6,204,437 DNA constructs and plants incorporating them
446 6,204,040 Gluconobacter suboxydans sorbitol dehydrogenase, genes and methods of use thereof
447 6,204,017 Polynucleotide encoding a histamine receptor
448 6,204,016 Tyrosylprotein sulfotransferases and methods of use thereof
449 6,200,811 Cell transformation vector comprising an HIV-2 packaging site nucleic acid and an HIV-1 GAG protein
450 6,200,803 Essential genes of yeast as targets for antifungal agents, herbicides, insecticides and anti-proliferative drugs

451 6,200,763 Myeloid cell leukemia associated gene mcl-1
452 6,200,749 Mutated forms of the ataxia-telangiectasia gene and method to screen for a partial A-T phenotype
453 6,198,020 Nitric oxide as an activator of the plant pathogen defense systems
454 6,197,925 NF-AT polypeptides and polynucleotides
455 6,197,517 Essential genes of yeast as targets for antifungal agents, herbicides, insecticides and anti-proliferative drugs
456 6,197,069 Adrenomedullin receptor polynucleotides
457 6,194,638 Alteration of hemicellulose concentration in plants
458 6,194,637 Maize DNA ligase I orthologue and uses thereof
459 6,194,547 ETS2 repressor factor (ERF)
460 6,191,260 Brain-associated inhibitor of tissue-type plasminogen activator
461 6,190,189 Cellulases and coding sequences
462 6,187,909 Viral encoded semaphorin protein receptor polypeptides
463 6,187,560 Polynucleotides and polypeptides belonging to the uncoupling proteins family
464 6,187,559 Phospholipase D gene
465 6,184,355 FAE1 genes and their uses
466 6,184,202 Cell death regulators
467 6,184,018 .beta.-glucosidase coding sequences and protein from orpinomyces PC-2
468 6,183,990 Compounds
469 6,183,988 Leukocyte-specific protein and gene, and methods of use thereof
470 6,183,961 Methods and compositions for regulating cell cycle progression
471 6,183,751 Unique associated Kaposi's Sarcoma virus sequences and uses thereof
472 6,180,850 Maize Ku70 orthologue and uses thereof
473 6,180,774 Synthetic DNA sequences having enhanced expression in monocotyledonous plants and method for preparation thereof
474 6,180,406 Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
475 6,180,112 Pasteurella haemolytica vaccine
476 6,177,611 Maize promoters
477 6,175,058 Nucleic acid sequence encoding FLP recombinase
478 6,174,689 Viral encoded semaphorin protein receptor DNA and polypeptides
479 6,174,676 Cytokine-stress- and oncoprotein-activated human protein kinase kinases
480 6,174,532 L2 immunogenic peptides of papillomavirus
481 6,174,528 Synthetic peptides and vaccines comprising same
482 6,172,211 Nucleic acid encoding tag7 polypeptide

483	6,171,833	Pyruvate carboxylase from corynebacterium glutamicum
484	6,171,820	Saturation mutagenesis in directed evolution
485	6,171,816	Human XAG-1 polynucleotides and polypeptides
486	6,171,781	NF-AT polypeptides and polynucleotides
487	6,171,590	Chemokine receptor peptide for inducing an immune response
488	6,169,073	Peptides and peptidomimetics with structural similarity to human p53 that activate p53 function
489	6,166,301	Method for assaying genetic attributes in cotton fiber cells
490	6,166,195	Nematode-active toxins and genes which code therefor
491	6,166,182	Human neurotensin receptor type 2 and splice variants thereof
492	6,166,178	Telomerase catalytic subunit
493	6,165,793	Methods for generating polynucleotides having desired characteristics by iterative selection and recombination
494	6,162,632	OxlT sequence and its use
495	6,159,469	Withdrawn
496	6,156,878	Ligand (ACT-4-L) to a receptor on the surface of activated CD4.sup.+ T-cells
497	6,156,310	Topoisomerase III
498	6,153,430	Nucleic acid encoding mesothelin, a differentiation antigen present on mesothelium, mesotheliomas and ovarian cancers
499	6,153,402	Death domain containing receptors
500	6,150,132	Chemokine receptor able to bind to MCP-1, MIP-1.alpha. and/or RANTES. Its uses

Evidence Appendix (c)

Description of BESTFIT

BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman.

BestFit inserts gaps to obtain the optimal alignment of the best region of similarity between two sequences, and then displays the alignment in a format similar to the output from Gap. The sequences can be of very different lengths and have only a small segment of similarity between them. You could take a short RNA sequence, for example, and run it against a whole mitochondrial genome.

BestFit is the most powerful method in the Wisconsin Package(TM) for identifying the best region of similarity between two sequences whose relationship is unknown.

BestFit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2; 482-489 (1981)) to find the best segment of similarity between two sequences. BestFit reads a scoring matrix that contains values for every possible GCG symbol match (see the LOCAL DATA FILES topic below) . The program uses these values to construct a path matrix that represents the entire surface of comparison with a score at every position for the best possible alignment to that point. The quality score for the best alignment to any point is equal to the sum of the scoring matrix values of the matches in that alignment, less the gap creation penalty times the number of gaps in that alignment, less the gap extension penalty times the total length of all gaps in that alignment. The gap creation and gap extension penalties are set by you. If the best path to any point has a negative value, a zero is put in that position. After the path matrix is complete, the highest value on the surface of comparison represents the end of the best region of similarity between the sequences. The best path from this highest value backwards to the point where the values revert to zero is the alignment shown by BestFit. This alignment is the best segment of similarity between the two sequences. See, http://www.biology.wustl.edu/gcg/bestfit.html#function.

Evidence Appendix (d)

List of 27 granted U.S. Patents and representative claims wherein "precent homology" and/or BESTFIT are recited

Debyser et al
Serial No. 09/403,625
Evidence Appendix (d)

U.S. Patent No. 5,871,969
Hastings , et al. February 16, 1999

United States Patent 5,968,780
Fan , et al. October 19, 1999

United States Patent 5,985,614
Rosen , et al November 16, 1999

United States Patent 5,998,171
Yu , et al. December 7, 1999

United States Patent 6,011,012
Ni , et al. January 4, 2000

United States Patent 6,027,916
Ni , et al. February 22, 2000

United States Patent 6,028,169
Kreider , et al. February 22, 2000

United States Patent 6,096,515
Crabtree , et al. August 1, 2000

United States Patent 6,130,079
Ni , et al. October 10, 2000

United States Patent 6,143,498
Olsen , et al. November 7, 2000

United States Patent 6,150,099
Crabtree , et al. November 21, 2000

United States Patent 6,171,781
Crabtree , et al. January 9, 2001

United States Patent 6,171,816
Yu , et al. January 9, 2001

United States Patent 6,174,532
Campo , et al. January 16, 2001

United States Patent 6,242,566
Godfrey ,et al. June 5, 2001

United States Patent 6,261,801
Wei , et al. July 17, 2001

United States Patent 6,284,486
Olsen , et al. September 4, 2001

United States Patent 6,329,568
Gonsalves , et al. December 11, 2001

United States Patent 6,388,052
Crabtree , et al. May 14, 2002

United States Patent 6,403,557
Greene , et al. June 11, 2002

United States Patent No. 6,562,593
Merkulov , et al. May 13, 2003

United States Patent No. 6,541,233
Hillen , et al. April 1, 2003

United States Patent No. 6,497,880
Wisniewski December 24, 2002

United States Patent 6,391,847
Evans , et al. May 21, 2002

United States Patent No. 6,093,535
Mori , et al. July 25, 2000

United States Patent No. 5,830,740
Miller , et al. November 3, 1998

United States Patent No. 5,695,960
Chan , et al. December 9, 1997

United States Patent No. 6,562,593
Merkulov , et al. May 13, 2003

Isolated human transporter proteins, nucleic acid molecules encoding human transporter proteins, and uses thereof

Appl. No.: **740041** Filed: **December 20, 2000**
The present application claims priority to provisional applications U.S. Ser. No. 60/251,035 filed Dec. 5, 2000.

Claim 17. An isolated nucleic acid molecule encoding a human transporter peptide, said nucleic acid molecule sharing at least 90 ***percent homology*** with a nucleic acid molecule shown in SEQ ID NO:1.

To determine the percent identity of two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% or more of a reference sequence is aligned for comparison purposes. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity and similarity between two sequences can be accomplished using a mathematical algorithm. (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J. Mol. Biol. (48):444-453 (1970)) algorithm which has been incorporated into the

GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (Devereux, J., et al., Nucleic Acids Res. 12(1):387 (1984)) (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Myers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against sequence databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (J. Mol. Biol. 215:403-10 (1990)). BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the proteins of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (Nucleic Acids Res. 25(17):3389-3402 (1997)). When utilizing BLAST and gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

Full-length pre-processed forms, as well as mature processed forms, of proteins that comprise one of the peptides of the present invention can readily be identified as having complete sequence identity to one of the transporter peptides of the present invention as well as being encoded by the same genetic locus as the transporter peptide provided herein.

Allelic variants of a transporter peptide can readily be identified as being a human protein having a high degree (significant) of sequence homology/identity to at least a portion of the transporter peptide as well as being encoded by the same genetic locus as the transporter peptide provided herein. Genetic locus can readily be determined based on the genomic information provided in FIG. 3, such as the genomic sequence mapped to the reference human. As indicated by the data presented in FIG. 3, the map position was determined to be on chromosome 12 by ePCR, and confirmed with radiation hybrid mapping. As used herein, two proteins (or a region of the proteins) have significant homology when the amino acid sequences are typically at least about 70-80%, 80-90%, and more typically

at least about 90-95% or more homologous. A significantly homologous amino acid sequence, according to the present invention, will be encoded by a nucleic acid sequence that will hybridize to a transporter peptide encoding nucleic acid molecule under stringent conditions as more fully described below.

United States Patent No. 6,541,233
Hillen , et al. April 1, 2003

.beta.-glucanase from a bacillus

Appl. No.: **463862**
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Jul 30, 1997[DE] 197 32 751

Claim 4. An isolated polynucleotide comprising the sequence SEQ ID NO:2 or a polynucleotide with more than 70 ***percent homology*** to SEQ ID NO:2, wherein said polynucleotide encodes a polypeptide with .beta.-glucanolytic activity.

Claim 5. The polynucleotide of claim 4 which is 75 to 99 percent homologous to the sequence reproduced in SEQ ID-NO:2.

Claim 7. An isolated polypeptide with .beta.-glucanolytic activity and a homology of more than 70 percent to the polypeptide with the amino acid sequence SEQ ID NO:1.

Claim 8. The polypeptide of claim 7 with a homology of 75 to 99 percent to the polypeptide with the amino acid sequence SEQ ID NO:1.

A .beta.-glucanase according to the invention preferably has a homology of more than 70%, more particularly 75% to 99%, to the .beta.-glucanase from Bacillus alkalophilus DSM 9956. The same applies to the basic gene.

United States Patent No. 6,497,880
Wisniewski December 24, 2002

Heat shock genes and proteins from Neisseria meningitidis, Candida glabrata and Aspergillus fumigatus

Appl. No.: **207388** Filed: **December 8, 1998**

Claim 2. An isolated polypeptide comprising an amino acid sequence that is at least 95% homologous to the protein encoded by SEQ ID NO:5, wherein the polypeptide comprises a peptide of at least 8 contiguous amino acids of the protein encoded by SEQ ID NO:5, wherein the peptide binds to a major histocompatibility complex molecule, and wherein ***percent homology*** is determined according to an algorithm incorporated in a protein database search program used in BLAST (BLAST.TM., a computer program) or DNA STAR MEGALIGN (DNA STAR MEGALIGN.TM., a computer program).

In certain embodiments, the isolated Hsp60 polypeptide is derived from proteolytic cleavage or chemical synthesis, or is an expression product of a transformed host cell containing a nucleic acid molecule encoding the Hsp60 or portion thereof. In further certain embodiments, the isolated Hsp60 polypeptide comprises greater than 95% homology to the Hsp60 polypeptide of FIG. 21, and the isolated Hsp60 polypeptide is able to be selectively bound by an antibody specific for a Candida glabrata Hsp60.

Within the context of this invention, it should be understood that Hsp70 and Hsp60 include wild-type/native protein sequences, as well as other variants (including alleles) and fragments of the native protein sequences. Briefly, such variants may result from natural polymorphisms or be synthesized by recombinant methodology or chemical synthesis, and differ from wild-type proteins by one or more amino acid substitutions, insertions, deletions, or the like. Further, in the region of homology to the native sequence, a variant should preferably have at least 95% amino acid sequence homology, and within certain embodiments, greater than 97% or 98% homology. As used herein, amino acid "homology" is determined by a computer algorithm incorporated in a protein database search program commonly used in the art, and more particularly, as incorporated in the programs BLAST (BLAST.TM., a computer program) (Altschul et al., Nucleic Acids Res. (25) 3389-3402, 1997) or DNA STAR MEGALIGN (DNA STAR MEGALIGN.TM., a computer program) which return similar results in homology calculations. As will be appreciated by those of ordinary skill in the art, a nucleotide sequence encoding an Hsp or a variant may differ from the native sequences presented herein due to codon degeneracies,

nucleotide polymorphisms, or nucleotide substitutions, deletions or insertions.

United States Patent 6,391,847
Evans , et al. May 21, 2002

Method, polypeptides, nucleotide sequence of XOR-6, a vitamin D-like receptor from xenopus

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PCT NO: **PCT/US96/00058**
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102(e) Date: **July 17, 1997**
PCT PUB.NO.: **WO96/22390**
PCT PUB. Date: **July 25, 1996**
This application is a filing under 35 U.S.C. .sctn.371 from PCT/US96/00058, filed Jan. 16, 1996; which is a continuation-in-part and claims priority to U.S. patent application Ser. No. 08/374,445; filed Jan. 17, 1995, now abandoned

Claim 14. A method of testing a compound for its ability to regulate transcription-activating effects of a nuclear receptor polypeptide wherein said receptor comprises a DNA binding domain having at least about 73 ***percent homology*** with residues 37-102 of the amino acid sequence of SEQ ID NO:2, said method comprising assaying for a change in expression of reporter protein upon contacting of test cells with said compound, as compared to the expression of said reporter protein in the absence of said compound, and identifying as a compound that regulates the transcription-activating effects of said receptor those which cause a change in expression of said reporter protein when compared to the expression of said reporter in the absence of said compound; wherein said test cell comprises said receptor polypeptide and reporter vector, wherein said reporter vector comprises:
(a) a promoter that is operable in said test cell,
(b) a hormone response element for said receptor, and
(c) DNA encoding said reporter protein,
wherein said reporter protein-encoding DNA is operatively linked to said promoter for transcription of said DNA,
wherein said promoter is operatively linked to said hormone response element for activation thereof by said receptor polypeptide.

Claim 16. A method according to claim 14, wherein said receptor further comprises: a ligand binding domain having at least about 42 percent homology to residues 183-386 of the amino acid sequence of SEQ ID NO:2.

As used herein, nucleotide sequences which are substantially the same share at least about 90% identity, and amino acid sequences which are substantially the same typically share more than 95% amino acid identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

FIG. 1 presents a schematic comparison between XOR-6 and the human vitamin D3 receptor. The two amino acid sequences were aligned using the program GAP (see Devereaux et al., in Nucl. Acids Res. 12:387-395 (1984)). Similarity between XOR-6 and hVDR is expressed as percent amino acid identity.

United States Patent No. 6,093,535
Mori , et al. July 25, 2000

Method for identifying attenuated chickenpox virus Oka strain or strain originating therein and acceptable as attenuated chickenpox vaccine virus

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Filed: **January 15, 1998**
PCT Filed: **May 15, 1997**
PCT NO: **PCT/JP97/01646**
371 Date: **January 15, 1998**
102(e) Date: **January 15, 1998**
PCT PUB.NO.: **WO97/43420**
PCT PUB. Date: **November 20, 1997**
Foreign Application Priority Data
May 15, 1996[JP] 8-158795
The application claims the benefit under 35 U.S.C. .sctn.371 of prior PCT International Application No. PCT/JP97/01646 which has an international filing date of May 15, 1997 which designated the United States of America, the entire contents of which are hereby incorporated by reference.

Claim 1. A method for identifying the attenuated varicella virus Oka strain or a strain derived therefrom which functions as an effective component of an attenuated variceila vaccine, which comprises:
analyzing a genomic DNA and DNA fragments thereof present in a sample of varicella virus;
determining whether the analyzed genomic DNA and DNA fragments thereof of said sample of varicella virus satisfy eight characteristics (A1) to (A8) defined below; and
identifying said sample of varicella virus as the attenuated varicella virus Oka strain or a strain derived therefrom which functions as an effective component of an attenuated varicella live vaccine when said sample satisfies all of the following eight characteristics (A1) to (A8):
(A1) the size of the K fragment obtained by digesting the varicella virus genomic DNA with the restriction enzyme HpaI is 3231 bp;
(A2) the size of the P fragment obtained by digesting the varicella virus genomic DNA with the restriction enzyme EcoRI is 1749 bp;
(A3) when a DNA fragment which is amplified from the varicella virus genomic DNA by PCR using the PCR primer 1 (SEQ ID NO. 3) and the PCR primer 3 (SEQ ID NO. 5) is treated with the restriction enzyme AccIII, the DNA fragment is cleaved into two parts having sizes of 1208 bp and 556 bp, respectively;
(A4) a DNA fragment which is amplified from the varicella virus genomic DNA by

PCR using the PCR primer 2 (SEQ ID NO. 4) and the PCR primer 3 (SEQ ID NO. 5) has a size of 487 bp;
(A5) the varicella virus genomic DNA and the attentuated varicella virus Oka strain genomic DNA exhibit substantially the same electrophoretic mobility with respect to a DNA fragment determined by PCR-SSCP wherein the PCR primer 2 (SEQ ID NO. 4) and the PCR primer 3 (SEQ ID NO. 5) are used in the PCR of said PCR-SSCP;
(A6) a DNA fragment which is amplified from the varicella virus genomic DNA by PCR using the PCR primer PS1 (SEQ ID NO. 7) and the PCR primer PS2 (SEQ ID NO. 8) lacks a restriction enzyme PstI cleavage site;
(A7) the homology between the 162 amino acid sequence coded by the 487 bp DNA fragment amplified from the varicella virus genomic DNA by PCR using the PCR primer 2 (SEQ ID NO. 4) and the PCR primer 3 (SEQ ID NO. 5), and the 162 amino acid sequence of SEQ ID NO. 1 is 98 to 100%, wherein the ***percent homology*** is represented by the following formula (1):
{(162-n)/162.times.100 (1)
wherein n represents the number of different amino acids; and
(A8) the homology between the 560 amino acid sequence coded by the entire coding region of Gene14, and the 560 amino acid sequence of SEQ ID NO. 2 is 99 to 100%, wherein the ***percent homology*** is represented by the following formula (2):
{(560-n)/560}.times.100 (2)
wherein n represents the number of different amino acids.

Further, the nucleotide sequence of each of the R2-487 regions was determined by Cycle Sequence Kit (manufactured and sold by TAKARA SHUZO Co. Ltd., Japan; Manual Code No. R014). The nucleotide sequences of the R2-487 regions of the samples were individually compared with the nucleotide sequence of the attenuated Oka strain to determine the DNA homology between the nucleotide sequences, and also, the amino acid sequences of the R2-487 regions of the samples which were obtained by translating the nucleotide sequences in accordance with the universal code were individually compared with that of the Oka strain to determine the homology between the amino acid sequences. The determination was performed by a computer software for gene analysis {GENETYX (ver. 9.0)(A computer Software for analyzing genes and proteins; manufactured and sold by Software Development Co., Ltd., Japan}. The nucleotide sequence and amino acid sequence of the R2-487 region of the attenuated Oka strain are shown in SEQ ID NO. 1.

The comparison between the nucleotide sequence of the R2-487 region of the attenuated Oka strain and the nucleotide sequence of each of the 10 samples including the Kawaguchi strain shows that a difference in nucleotides was found with respect to 4 or more nucleotides in the R2-487 region of each of the

samples, and that this difference in the nucleotides was accompanied by a change in amino acids (coded by 162 codons, i.e., 162 codons=487 bp/3). Thus, the homology between the amino acid sequence of the attenuated Oka strain and that of each of the 10 samples was less than 98% {i.e., 487 bp/3=162 codons; [(162 codons-4 different codons)/162 codons].times.100=97.5% <98%}.

United States Patent No. 5,830,740
Miller , et al. November 3, 1998

Serine protease operative between 75.degree.C. and 103.degree.C.

Appl. No.: **278042** Filed: **July 20, 1994**

Claim 4. An isolated and purified serine protease which exhibits proteolytic activity at temperature of between about 75.degree. C. and about 130.degree. C., said protease comprising an amino acid sequence having at least 90 ***percent homology*** to SEQ ID NO:3.

Claim 5. An isolated and purified serine protease which exhibits proteolytic activity at temperature of between about 75.degree. C. and about 130.degree. C., said protease comprising an amino acid sequence having at least 90 ***percent homology*** to SEQ ID NO:2.

FIGS. 5A, 5B and 5C are pictorial representations of three-dimensional models of aerolysin. The models were built using the Biosyn Homology program with the tertiary structure of the thermitase as a starting point. Residue numbering follows equivalent sites in the P. aerophilum sequence. Numbering of secondary structure elements is from FIGS. 6 and 7. FIG. 5A shows clustering of thermophilic residues from two surface loops L1 and L3; FIG. 5B shows thermophilic sites in two adjacent extended strands E6 and E7 linked by loop L8; and FIG. 5C shows thermophilic sites on each side of surfaces helices III and IV.

The translated amino acid sequence (FIG. 1 and sequence ID No. 2) shows a long open reading frame starting 83 amino acids upstream of sequence homology to various subtilisins. The first 15 amino acids encoded by this region showed similarities to leader sequences from subtilisins Carlsberg, BPN', 1168 and T. aquaticus aqualysin I. The intervening region is not homologous to any known protein and appears to be the N-terminal peptide autocatalytically cleaved on subtilisin's export from the cell (Terada et al. 1990). The amino acid sequence of the proteolytically active enzyme remaining after cleavage is set forth in sequence ID No. 3. Based on these considerations, methionine was assigned as the initiator for the protein, as well as a potential cleavage site for the mature protein. The end of the gene is clearly defined by 5 stop codons. The first stop codon appeared 15 bases upstream, comparable in position to the end of the gene in several Bacillus species, and was followed by a poly T region.

The alignment similarity scores identified the P. aerophilum sequence as most similar to Gram-positive subtilisins, but PredictProtein identified thermitase from Thermoactinomyces vulgaris (16) as having the most similar structure. Similarity

to other serine proteases was much weaker. In particular, the P. aerophilum sequence showed weak homology to aqualysin I. produced by Thermus aquaticus (17), and halolysin, a serine protease from a moderately thermophilic (60.degree. C.) and halophilic archaeum (18). Neutral proteases such as thermolysin (19), despite their structural similarity, were not recovered by BLAST or PredictProtein, and were not included in the alignment.

The above multiple sequence alignment of aerolysin with 14 different serine type proteases shows that subtilisins from Gram-positive bacteria, rather than archaeal or eukaryal serine proteases, have the greatest homology. In view of the above demonstrated relationship of aerolysin to subtilisins, aerolysin will be useful in the same type of applications in which these other subtilisins and serine proteases are presently being used.

United States Patent No. 5,695,960
Chan , et al. December 9, 1997

Hippuricase gene

Appl. No.: **485216**
Filed: **June 7, 1995**
This application is continuation of PCT/CA94/00270, filed May 13, 1994, which is a continuation-in-part of U.S. Ser. No. 08/061,696, filed May 14, 1993, now abandoned.

Claim 5. A method for preparing hippuricase comprising:
(a) transferring a recombinant expression vector containing a nucleic acid molecule encoding a polypeptide having the amino acid sequence and enzymatic activity of Campylobacter jejuni hippuricase into a host cell; (b) selecting transformed host cells from untransformed host cells; (c) culturing a selected transformed host cell under conditions which allow expression of the polypeptide; and (d) isolating the polypeptide.

Claim 6. A method according to claim 5 wherein said polypeptide has the amino acid sequence as shown in the Sequence Listing as SEQ ID NO:1, or a sequence having between 97 and 100 ***percent homology*** thereto, having the enzymatic activity of Campylobacter jejuni hippuricase.

Claim 7. A method according to claim 5 wherein said nucleic acid comprises (a) a nucleic acid sequence as shown in SEQ ID:1 and FIG. 1, wherein T can also be U; (b) nucleic acid sequences complementary to (a); or (c) nucleic acid sequences which are at least 85% homologous to (a).

The invention still further provides a purified and isolated polypeptide having part or all of the primary structural confirmation (ie. continuous sequence of amino acid residues) and the enzymatic activity of hippuricase. In a preferred embodiment the polypeptide has an amino acid sequence as shown in FIG. 1 and in the Sequence Listing as SEQ ID NO:1 and NO:2, or a sequence having between 97 and 100 percent homology thereto.

It will be appreciated that the invention includes nucleotide or amino acid sequences which have substantial sequence homology with the nucleotide and amino acid sequences shown in the Sequence Listing as SEQ ID NO:1 and NO:2. The term "sequences having substantial sequence homology" means those nucleotide and amino acid sequences which have slight or inconsequential sequence variations from the sequences disclosed in the Sequence Listing as SEQ ID NO:1 and NO:2 i.e. the homologous sequences function in substantially

the same manner to produce substantially the same polypeptides as the actual sequences. The variations may be attributable to local mutations or structural modifications. It is expected that a sequence having 85-90% sequence homology with the DNA sequence of the invention will provide a functional hippuricase polypeptide.

Nucleic acid sequences having substantial sequence homology include nucleic acid sequences having at least 85%, preferably at least 90% homology with the nucleic acid sequence as shown in SEQ. ID. NO:1 and in FIG. 1; and fragments thereof having at least 15 to 30, preferably at least 15 bases, most preferably 20 to 30, which will hybridize to these sequences under stringent hybridization conditions. Stringent hybridization conditions are those which are stringent enough to provide specificity, reduce the number of mismatches and yet are sufficiently flexible to allow formation of stable hybrids at an acceptable rate. Such conditions are known to those skilled in the art and are described, for example, in Sambrook, et al, (1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor). By way of example only, stringent hybridization with short nucleotides may be carried out at 5.degree.-10.degree. below the T.sub.m using high concentrations of probe such as 0.01-1.0 pmole/ml.

U.S. Patent No. 5,871,969
Hastings , et al. February 16, 1999

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a member selected from the group consisting of:
(a) a nucleotide sequence encoding a full-length Neuronal Attachment Factor-1 (NAF-1) polypeptide having the complete amino acid sequence in SEQ ID NO:2, or the complete amino acid sequence encoded by the cDNA clone contained in the ATCC Deposit No. 97343;
(b) a nucleotide sequence encoding amino acids 2 to 331 (SEQ ID NO:2) of a full-length NAF-1 polypeptide or the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone contained in the ATCC Deposit No. 97343;
(c) a nucleotide sequence encoding a predicted mature form of the NAF-1 polypeptide having the amino acid sequence at positions 24 to 331 in SEQ ID NO:2 or as encoded by the cDNA clone contained in the ATCC Deposit No. 97343;
(d) a nucleotide sequence encoding a predicted mature form of the NAF-1 polypeptide having the amino acid sequence at positions 27 to 331 in SEQ ID NO:2 or as encoded by the cDNA clone contained in the ATCC Deposit No. 97343; and
(e) a nucleotide sequence fully complementary to any of the nucleotide sequences in (a), (b), (c) or (d) above;
wherein percentage of identity is determined using the Bestfit program with parameters set such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and gaps of up to 5% of the total number of nucleotides in the reference sequence are allowed, and wherein up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in FIG. 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 5371 1). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best

segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in FIG. 1 (SEQ ID NO:1) or to the nucleic acid sequence of the deposited CDNA, irrespective of whether they encode a polypeptide having NAF-1 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having NAF-1 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having NAF-1 activity include, inter alia, (1) isolating the NAF-1 gene or allelic variants thereof in a CDNA library; (2) in situ hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the NAF-1 gene, as described in Verma et al., Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988); and Northern Blot analysis for detecting NAF-1 mRNA expression in specific tissues.

United States Patent 5,968,780
Fan , et al. October 19, 1999

This application claims benefit of 35 U.S.C. section 119(e) based on copending U.S. Provisional Application Serial No. 60/038,829, filed Feb. 6, 1997, which is hereby incorporated herein by reference.

1. An isolated nucleic acid molecule comprising a first polynucleotide sequence 95% or more identical to a second polynucleotide sequence selected from the group consisting of:
(a) a polynucleotide sequence encoding amino acids -26 to 485 of SEQ ID NO:2;
(b) a polynucleotide sequence encoding amino acids -25 to 485 of SEQ ID NO:2;
(c) a polynucleotide sequence encoding amino acids 1 to 485 of SEQ ID NO:2; and
(d) a polynucleotide sequence complementary to any of the polynucleotide sequences in (a), (b) or (c) above;
wherein percentage identity is determined using the BESTFIT program with parameters that calculate identity over the full length of the second polynucleotide sequence and that allows gaps of up to 5% of the total number of nucleotides of said nucleotide sequence.

The DCDGF protein of the present invention shares sequence homology with the translation product of the insect Sarcophaga perigrina (flesh fly) mRNA for Insect-Derived Growth Factor (IDGF) (see FIG. 2; SEQ ID NO:3). Thus, the complete DCDGF amino acid sequence of SEQ ID NO:2 shares about 38.0% identity and about 58.2% similarity with the amino acid sequence encoded by the insect mRNA for IDGF (Homma et al., supra, which can be accessed on GenBank as Accession No. D83125), as determined by analysis with Bestfit (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711) using the default parameters (see FIG. 2). The shared homology includes the conserved cysteines at positions 108 and 133 in SEQ ID NO:2, which also are conserved in the atrial gland granule-specific antigen (AGSA) of Aplysia californica, as reported by K. Homma et al., supra. IDGF is present throughout embryonic development and stimulates proliferation of embryonic insect cells and therefore is thought to be important in development of the insect from the fertilized egg. The homology between IDGF and DCDGF, as well as the facts that DCDGF is produced by dendritic cells, which activate T cells, and the gene for DCDGF has been mapped to a locus on chromosome 22 which is associated with DiGeorge Syndrome, all indicate involvement of DCDGF in early stages of human development and developmentally related pathologies including, for

instance, DiGeorge Syndrome, as well as in immune system disorders, particularly relating to cellular immunity.

FIG. 2 shows the regions of identity between the amino acid sequences of the DCDGF protein (SEQ ID NO:2) and the translation product of the insect mRNA for IDGF (SEQ ID NO:3), determined by the computer program Bestfit (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711) using the default parameters.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in FIG. 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

United States Patent 5,985,614
Rosen , et al. November 16, 1999

This application claims the benefit of the filing date of provisional application No. 60/024,882 filed on Aug. 30, 1996, which is herein incorporated by reference.

5. The isolated nucleic acid molecule of claim 1, wherein said polypeptide sequence is (d).

6. The isolated nucleic acid molecule of claim 1, wherein said polypeptide sequence is (e).

7. The isolated nucleic acid molecule of claim 1, wherein the number of amino acid substitutions is one to three.

8. An isolated nucleic acid molecule comprising a first polynucleotide sequence at least 95% identical to a second polynucleotide sequence, wherein said second polynucleotide sequence is selected from the group consisting of:
(a) a polynucleotide sequence encoding amino acids -24 to 153 of SEQ ID NO:2;
(b) a polynucleotide sequence encoding amino acids -23 to 153 of SEQ ID NO:2;
(c) a polynucleotide sequence encoding amino acids 1 to 153 of SEQ ID NO:2;
(d) a polynucleotide sequence encoding the same amino acid sequence as encoded by the cDNA clone contained in ATCC Deposit No. 97662;
(e) a polynucleotide sequence encoding the mature interleukin-19 (IL-19) polypeptide having the amino acid sequence
encoded by the cDNA clone contained in ATCC Deposit No. 97662;
(f) a polynucleotide sequence encoding amino acids -5 to 4 of SEQ ID NO:2;
(g) a polynucleotide sequence encoding amino acids 64 to 82 of SEQ ID NO:2;
(h) a polynucleotide sequence encoding amino acids 115 to 125 of SEQ ID NO:2; and
(i) the complement of (a), (b), (c), (d), (e), (f), (g) or (h);
wherein % identity is calculated using BESTFIT with the parameters set such that % identity is calculated over the full length of said second polynucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in said
second polynucleotide sequence are allowed.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in FIG. 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. Bestfit uses the local homology algorithm of Smith and

Waterman (Advances in Applied Mathematics 2: 482-489, 1981) to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

United States Patent 5,998,171
Yu , et al. December 7, 1999

This application claims priority benefit to U.S. application Ser. No. 60/024,058, filed Aug. 16, 1996, which disclosure is herein incorporated by reference.

90. An isolated polynucleotide comprising a first nucleotide sequence 95% or more identical to a second nucleotide sequence selected from the group consisting of:
(a) a nucleotide sequence encoding amino acids 1 to 169 of SEQ ID NO:2;
(b) a nucleotide sequence encoding amino acids 2 to 169 of SEQ ID NO:2; and
(c) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97640;
wherein percentage identity is determined using the BESTFIT program with parameters that calculate identity over the full length of said second nucleotide sequence and that allow gaps of up to 5% of the total number of nucleotides of said second nucleotide sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98%, or 99% identical to, for instance, the nucleotide sequence shown in FIGS. 1A-1B or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2:482-489 (1981), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

United States Patent 6,011,012
Ni , et al. January 4, 2000

The present application is a continuation-in-part application of co-pending U.S. patent application Ser. No. 08/461,030, filed Jun. 5, 1995.

1. An isolated polypeptide having cysteine protease inhibiting activity comprising an amino acid sequence selected from the group consisting of:
(a) the amino acid sequence no of SEQ ID NO:2 wherein n is any integer 1-35 and c is any integer 143-149;
(b) amino acid sequence at least 95% identical to the amino acid sequence of (a) as determined by the Bestfit computer program using default parameters;
(c) the amino acid sequence of a fragment of SEQ ID NO:2;
(d) an amino acid sequence at least 95% identical to the amino acid sequence of (c) as determined by the Bestfit computer program using default parameters;
(e) the amino acid sequence of a fragment of the polypeptide encoded by the human cDNA contained in ATCC Deposit No. 97156; and
(f) an amino acid sequence at least 95% identical to the amino acid sequence of (e) as determined by the Bestfit computer program using default parameters.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in FIG. 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

United States Patent 6,027,916
Ni , et al. February 22, 2000

This application claims the benefit of the filing date of provisional application 60/028,093 filed on Oct. 9, 1996, which is herein incorporated by reference.

1. An isolated nucleic acid molecule comprising a polynucleotide which encodes an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of:
(a) amino acids 1 to 311 of SEQ ID NO:4;
(b) amino acids 2 to 311 of SEQ ID NO:4;
(c) amino acids 1 to 200 of SEQ ID NO:8;
(d) amino acids 2 to 200 of SEQ ID NO:8;
(e) the human Galectin 9 amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97733; and
(f) the human Galectin 10SV amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97734;
wherein said 95% identity is determined using the Bestfit program having parameters set such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in FIGS. 1, 2A-2B, 3A-3B, or 4A-4B (SEQ ID NOs: 1, 3, 5, or 7) or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981)), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in FIGS. 1, 2A-2B, 3A-3B, or 4A-4B (SEQ ID NOs:2, 4, 6, or 8, respectively) or to

the amino acid sequence encoded by one of the deposited cDNA clones (ATCC Deposit Numbers 97732, 97733 and 97734) can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

United States Patent 6,028,169
Kreider , et al. February 22, 2000

This application claims benefit of the filing date of provisional application 60/042,269 filed Mar. 31, 1997, which is herein incorporated by reference.

1. An isolated polypeptide selected from the group consisting of:
(a) a polypeptide comprising the amino acid sequence of residue 4 to residue m in SEQ ID NO:2, wherein m is any one of residues 48-93 of SEQ ID NO:2, and wherein said polypeptide inhibits the Chemokine Receptor-3 (CCR3) signaling pathway;
(b) a polypeptide comprising the amino acid sequence of (a) except for one or more conservative amino acid substitutions, wherein said polypeptide inhibits the Chemokine Receptor-3 (CCR3) signaling pathway; and
(c) a polypeptide comprising a sequence at least 80% identical to the amino acid sequence of (a), wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the fall length of sequence (a) and that allow gaps in homology of up to 20% of the total number of residues in sequence (a), and wherein said polypeptide inhibits the Chemokine Receptor-3 (CCR3) signaling pathway.

FIG. 2 illustrates a comparison of the amino acid sequence homology between the polypeptide of the present invention with human MCP-1 (SEQ ID NO:5). Ck.beta.-6 shows 36% identity and 52% similarity with human MCP-1 as determined by the computer program Bestfit.

The polynucleotide of this invention was discovered from an activated monocyte cDNA library. It contains an open reading frame encoding a protein of approximately 119 amino acids in length of which the first 26 amino residues comprise a putative leader sequence. The mature protein therefore is predicted to be 93 amino acids in length. It is structurally related to mouse monocyte chemotactic protein-1 (MCP-1 or JE, sequence not shown), and human MCP-1 (SEQ ID NO:5) showing 36% identity, and 52% simlarity over the entire human MCP-1 protein sequence as determined by the computer program Bestfit (shown in FIG. 2). The polypeptide contains all four cysteine residues that occur in all chemokines in a characteristic motif. The spacing between these cysteines is conserved compared with the human MCP-1 and murine MCP-1/JE which strongly suggests that the new gene is a chemokine.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide

sequence shown in FIG. 1, or to the nucleotide sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in FIG. 1 (SEQ ID NO:2) or to the amino acid sequence encoded by the deposited cDNA clone can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the fall length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

United States Patent 6,096,515
Crabtree , et al. August 1, 2000

Filed: March 9, 1998

12. The isolated polynucleotide of claim 1, which encodes a polypeptide comprising an amino acid sequence that is at least 90% identical to 20 or more consecutive amino acids of the sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

14. The isolated polynucleotide of claim 1, which encodes a polypeptide comprising a Rel Similarity Region having an amino acid sequence which is at least about 73% identical to the amino acid sequence set forth in SEQ ID NO: 51, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

18. An isolated polynucleotide encoding a polypeptide comprising an amino acid sequence which is at least 90% identical to 20 or more consecutive amino acids of the amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

28. An isolated polynucleotide comprising a nucleotide sequence which is at least 73% identical to the nucleotide sequence set forth in SEQ ID NO: 45, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

29. The isolated polynucleotide of claim 28, comprising a nucleotide sequence which is at least about 90% identical to the nucleotide sequence set forth in SEQ ID NO: 45, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and

TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity).

United States Patent 6,130,079
Ni , et al. October 10, 2000

This application claims the benefit of the filing date of provisional application Ser. No. 60/033,868 filed on Dec. 20, 1996, which is herein incorporated by reference.

1. An isolated polynucleotide comprising a nucleotide sequence encoding an amino acid sequence at least 95% identical to amino acids 2 to 199 of SEQ ID NO:2,
wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of amino acids 2 to 199 of SEQ ID NO:2 and that allow gaps of up to 5% of the total number of residues in amino acids 2 to 199 of SEQ ID NO:2;
wherein said polynucleotide encodes a polypeptide which either induces apoptosis or generates antibody that binds the full length RAIDD protein

4. The isolated polynucleotide of claim 1, wherein said amino acid sequence is at least 95% identical to amino acids 1 to 199 of SEQ ID NO:2;
wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of amino acids 1 to 199 of SEQ ID NO:2 and that allow gaps of up to 5% of the total number of residues in amino acids 1 to 199 of SEQ ID NO:2.

17. The isolated polynucleotide of claim 15, wherein said amino acid sequence is at least 95% identical to the complete amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97824;
wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of the complete amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97824 and that allow gaps of up to 5% of the total number of residues of the complete amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97824.

70. An isolated polynucleotide comprising a nucleotide sequence encoding an amino acid sequence at least 95% identical to a reference amino acid sequence selected from the group consisting of:
(a) amino acids 8 to 80 of SEQ ID NO:2;
(b) amino acids 123 to 194 of SEQ ID NO:2;
(c) amino acids 1 to 117 of SEQ ID NO:2; and
(d) amino acids 95 to 199 of SEQ ID NO:2;
wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of the reference amino acid sequence and that allow gaps of up to 5% of the total number of residues of the reference amino acid sequence;

wherein said polynucleotide encodes a polypeptide which either induces apoptosis or generates antibody that binds the full length RAIDD protein.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to those described above can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

United States Patent 6,143,498
Olsen , et al. November 7, 2000

This application claims the benefit of the filing date of provisional application Ser. No. 60/046,415 filed on May 14, 1997, which is herein incorporated in its entirety.

1. An isolated polynucleotide comprising a nucleic acid at least 95% identical to a reference nucleic acid encoding amino acids 1 to 41 of SEQ ID NO:2, wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference nucleic acid and that allow gaps of up to 5% of the total number of nucleotides of said reference nucleic acid.

15. An isolated polynucleotide comprising a nucleic acid at least 95% identical to a reference nucleic acid encoding the mature amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97982, wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference nucleic acid and that allow gaps of up to 5% of the total number of nucleotides of said reference nucleic acid.

25. An isolated polynucleotide comprising a nucleic acid encoding an amino acid sequence at least 95% identical to amino acids 1 to 41 of SEQ ID NO:2 (the reference sequence), wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference sequence and that allow gaps of up to 5% of the total number of residues of said reference sequence.

36. An isolated polynucleotide comprising a nucleic acid encoding an amino acid sequence at least 95% identical to the mature amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97982 (the reference sequence), wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference sequence and that allow gaps of up to 5% of the total number of residues of said reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO: 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for

Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

United States Patent 6,150,099
Crabtree , et al. November 21, 2000

This application is a continuation application of Ser. No. 08/260,174 filed on Jun. 13, 1994, which is a continuation-in-part of U.S. Ser. No. 08/124,981 filed Sep. 20, 1993 (U.S. Pat. No. 5,837,840) which is a continuation-in-part of U.S. Ser. No. 07/749,385, filed Aug. 22, 1991 (U.S. Pat. No. 5,989,810).

5. The method of claim 1, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least about 80% identical to an amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights

16. The method of claim 15, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least about 80% similar to an amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

21. The method of claim 20, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least about 80% similar to an amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

25. The method of claim 23, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least about 80% identical to an amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin (genetics Software Package Release 7.0, using default gap weights.

29. The method of claim 27, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least about 80% identical to an amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

35. The method of claim 33, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least about 80% identical to an amino acid sequence set forth in SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

38. A method for identifying a compound which modulates the activity of an NF-AT polypeptide, comprising
(i) contacting an isolated NF-AT polypeptide or portion thereof sufficient for interacting with a molecule, with the molecule and a compound in conditions under which, but for the presence of the compound, the NF-AT polypeptide or portion thereof and the molecule interact, wherein the NF-AT polypeptide comprises at least 25 amino acids having an amino acid sequence which is at least 80% identical to an amino acid sequence of SEQ ID NO: 38, wherein the percent identity is determined with the algorithm (GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights; and
(ii) determining the level of interaction between the NF-AT polypeptide or portion thereof and the molecule in the presence relative to the absence of the compound,
such that a difference in the level of interaction between the NF-AT polypeptide or portion thereof and the molecule in the presence relative to the absence of the compound indicates that the compound modulates the activity of an NF-AT polypeptide.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

Additionally, computerized comparison of sequences shown in FIG. 1 to existing sequence databases can identify sequence motifs and structural conformations found in other proteins or coding sequences that indicate similar domains of the NF-AT.sub.c protein. For example but not for limitation, the programs GAP,

BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics computer Group, 575 Science Dr., Madison, Wis.) can be used to identify sequences in databases, such as GenBank/EMBL, that have regions of homology with a NF-AT.sub.c sequences. Such homologous regions are candidate structural or functional domains. Alternatively, other algorithms are provided for identifying such domains from sequence data.

United States Patent 6,171,781
Crabtree , et al. January 9, 2001

This application is a continuation-in-part of application Ser. No. 08/260,174, entitled "NF-AT Polypeptides and Polynucleotides", filed Jun. 13, 1994, which is a continuation-in-part of application Ser. No. 08/124,981, entitled "NF-AT Polypeptides and Polynucleotides", filed Sep. 20, 1993, U.S. Pat. No. 5,837,840. These applications are hereby incorporated by referenced herein.

42. A method for identifying a compound which modulates translocation of an NF-AT polypeptide across the nuclear membrane of a cell by binding of the compound to the NF-AT polypeptide, comprising
(i) contacting test compounds with an NF-AT polypeptide, or portion thereof, wherein the NF-AT polypeptide comprises at least 25 contiguous amino acids having an amino acid sequence which is at least 80% identical to an amino acid sequence of SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights;
(ii) identifying those test compounds which bind to the NF-AT polypeptide; and determining which of the test compounds so identified modulates translocation of an NF-AT polypeptide across the nuclear membrane of a cell.

60. A method for identifying a compound which promotes or inhibits translocation of an NF-AT polypeptide across the nuclear membrane of a cell, comprising

(i) providing a polypeptide complex comprising a nuclear localization sequence (NLS) of an NF-AT polypeptide and a portion of an NF-AT polypeptide which binds to said NLS, wherein the NF-AT polypeptide comprises at least 25 contiguous amino acids having an amino acid sequence which is at least 80% identical to an amino acid sequence of SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights;
(ii) contacting the polypeptide complex with test compounds and determining whether a test compound modulates the binding of the NLS to the portion of an NF-AT polypeptide which binds to said NLS; and
(iii) determining which of the test compounds so identified promotes or inhibits translocation of an NF-AT polypeptide across the nuclear membrane of a cell.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of

Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

As applied to polypeptides, a degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e. structurally related, at positions shared by the amino acid sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than 25% identity, with one of the NF-ATc sequences of the present invention. The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

Additionally, computerized comparison of sequences shown in FIG. 1 to existing sequence databases can identify sequence motifs and structural conformations found in other proteins or coding sequences that indicate similar domains of the NF-AT.sub.c protein. For example but not for limitation, the programs GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, 575 Science Dr., Madison, Wis.) can be used to identify sequences in databases, such as GenBank/EMBL, that have regions of homology with a NF-AT.sub.c sequences. Such homologous regions are candidate structural or functional domains.

United States Patent 6,171,816
Yu , et al. January 9, 2001

This application claims priority benefit to U.S. application Ser. No. 60/024,347, filed Aug. 23, 1996, which disclosure is herein incorporated by reference.

86. An isolated polynucleotide comprising a nucleic acid at least 95% identical to a reference nucleic acid encoding amino acids 1 to 155 of SEQ ID NO:2, wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference nucleic acid and that allow gaps of up to 5% of the total number of nucleotides of said reference nucleic acid.

100. An isolated polynucleotide comprising a nucleic acid at least 95% identical to a reference nucleic acid encoding the mature amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97641, wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference nucleic acid and that allow gaps of up to 5% of the total number of nucleotides of said reference nucleic acid.

111. An isolated polynucleotide comprising a nucleic acid encoding an amino acid sequence at least 95% identical to a reference amino acid sequence consisting of amino acids 1 to 155 of SEQ ID NO:2, wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference amino acid sequence and that allow gaps of up to 5% of the total number of residues of said reference amino acid sequence.

124. An isolated polynucleotide comprising a nucleic acid encoding an amino acid sequence at least 95% identical to a reference amino acid sequence consisting of the mature amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 97641, wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said reference amino acid sequence and that allow gaps of up to 5% of the total number of residues of said reference amino acid sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 or to the nucleotides sequence of the deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University

Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 or to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

United States Patent 6,174,532
Campo , et al. January 16, 2001

Appl. No.: 817548
Filed: May 12, 1997
PCT Filed: October 6, 1995
PCT NO: PCT/GB95/02372
371 Date: May 12, 1997
102(e) Date: May 12, 1997
PCT PUB.NO.: WO96/11273
PCT PUB. Date: April 18, 1996

1. An immunogenic peptide for the treatment of papillomavirus infection, wherein the peptide is: (a) a peptide from 10-30 amino acid residues in length having a sequence corresponding to a sequence from the N terminal amino acids 11-200 of papillomavirus L2 protein, (b) a peptide of 10-30 amino acid residues in length with at least 30% identity with the sequence from (a) as determined using the BESTFIT program, or (c) a peptide as defined in either (a) or (b) which is conjugated or fused to a protein or peptide other than a papillomavirus L2 protein or peptide.

Naturally, the skilled addressee will appreciate that there are computer programs available in the art which are able to make alignments between different amino acid sequences. An example of such a program is BESTFIT of TRANSLATE in the Genetic Computer Group (University of Wisconsin) package.

Examples of at least 10 amino acid residue long peptide sequences and of 14 amino acid long peptide sequences which can be optimally aligned using an appropriate computer program as described above (e.g. Bestfit of Translate) and are capable of being included in a peptide, polypeptide or protein format capable of an immunogenic potential include HPV-18, HPV-16, HPV-11 and HPV-6 as shown hereinbelow:

THR ASP PRO SER ILE VAL THR LEU ILE GLU HPV-18 (SEQ ID NO.4)
SER ASP PRO SER ILE VAL SER LEU VAL GLU HPV-16 (SEQ ID NO.5)
SER ASP PRO SER ILE VAL SER LEU ILE GLU HPV-11 (SEQ ID NO.6)
SER ASP PRO SER ILE VAL SER LEU ILE GLU HPV-6 (SEQ ID NO.7)
SER ASP PRO SER ILE VAL SER LEU ILE GLU CONSENSUS (SEQ ID NO.8)
HPV-18 ILE THR SER ALA GLY THR THR THR PRO ALA VAL LEU ASP ILE (SEQ ID NO.9)

HPV-16 ILE THR THR SER THR ASP THR THR PRO ALA ILE LEU ASP ILE (SEQ ID NO.10)

HPV-11 ILE THR SER SER GLU SER THR THR PRO ALA ILE LEU ASP VAL (SEQ ID NO.11)

HPV-6 ILE THR SER SER GLU THR THR THR PRO ALA ILE LEU ASP VAL (SEQ ID NO.12)

CONSENSUS

ILE THR SER SER GLU THR THR THR PRO ALA ILE LEU ASP VAL (SEQ ID NO.13)

United States Patent 6,242,566
Godfrey ,et al. June 5, 2001

Filed: February 10, 1994

28. An isolated ACT-4-L ligand polypeptide comprising an amino acid sequence that is a variant of the amino acid sequence of the extracellular domain of the ACT-4-L polypeptide shown in FIG. 10 (SEQ ID NO: 4), said variant having at least about 80% sequence identity to the amino acid sequence of the extracellular domain, said extracellular domain having an amino terminus of amino acid 51 of the amino acid sequence shown in FIG. 10 (SEQ ID NO: 4) and a carboxyl terminus of amino acid 183 of the amino acid sequence shown in FIG. 10 (SEQ ID NO: 4), said polypeptide having the ability to bind specifically to the ACT-4-h-1 receptor shown in FIG. 5 (SEQ ID NO: 2).

34. The isolated ACT-4-L ligand polypeptide of claim 28 wherein said sequence identity is determined by use of a BESTFIT homology algorithm with default gap weights.

35. An isolated ACT-4-L ligand soluble polypeptide comprising an amino acid sequence that has at least about 80% sequence identity to the amino acid sequence of the extracellular domain of the ACT-4-L polypeptide shown in FIG. 10 (SEQ ID NO: 4), said sequence identity being determined by use of a BESTFIT homology algorithm with default gap weights, said extracellular domain having an amino terminus of amino acid 51 of the amino acid sequence shown in FIG. 10 (SEQ ID NO: 4) and a carboxyl terminus of amino acid 183 of the amino acid sequence shown in FIG. 10 (SEQ ID NO: 4), said soluble polypeptide having the ability to bind specifically to the ACT-4-h-1 receptor shown in FIG. 5 (SEQ ID NO: 2).

37. An isolated variant ACT-4-L ligand polypeptide comprising an amino acid sequence that is a variant of the amino acid sequence shown in FIG. 10 (SEQ ID NO: 4) and which has at least about 80% sequence identity to the amino acid sequence shown in FIG. 10 (SEQ ID NO: 4), said sequence identity being determined by use of a BESTFIT homology algorithm with default gap weights, said polypeptide having the ability to bind specifically to the ACT-4-h-1 receptor shown in FIG. 5 (SEQ ID NO: 2).

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J.

Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. (USA) 85:2444 (1988), by computerized implementations of these algorithms (FASTDB (Intelligenetics), BLAST (National Center for Biomedical Information) or GAP, BESTFIT, FASTA, and TFASTA (Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.)), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs BLAZE (Intelligenetics) GAP or BESTFIT using default gap weights, share at least 70 percent or 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

United States Patent 6,261,801
Wei , et al. July 17, 2001

This application claims benefit of 35 U.S.C. section 119(e) based on U.S. Provisional Application Ser. No. 60/035,496, filed Jan. 14, 1997 and No. 60/054,885, filed Aug. 7, 1997.

1. An isolated nucleic acid molecule comprising a first polynucleotide sequence 95% or more identical to a second polynucleotide sequence selected from the group consisting of:
(a) a polynucleotide sequence encoding amino acids -26 to 233 of SEQ ID NO:2;
(b) a polynucleotide sequence encoding amino acids -25 to 233 of SEQ ID NO:2;
(c) a polynucleotide sequence encoding amino acids 1 to 233 of SEQ ID NO:2; and
(d) a polynucleotide sequence complementary to any of the polynucleotide sequences in (a), (b) or (c) above;
wherein percentage identity is determined using the BESTFIT program with parameters that calculate identity over the full length of the second polynucleotide sequence and that allows gaps of up to 5% of the total number of nucleotides of said nucleotide sequence.

21. An isolated nucleic acid molecule comprising a first polynucleotide sequence 95% or more identical to a second polynucleotide sequence selected from the group consisting of:
(a) a polynucleotide sequence encoding a TRID (TRAIL receptor without intracellular domain) polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97798;
(b) a polynucleotide sequence encoding the mature TRID polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97798; and
(c) a polynucleotide sequence complementary to any of the polynucleotide sequences in (a) or (b) above;
wherein percentage identity is determined using the BESTFIT program with parameters that calculate identity over the full length of the second polynucleotide sequence and that allows gaps of up to 5% of the total number of nucleotides of said nucleotide sequence.

38. An isolated nucleic acid molecule comprising a first polynucleotide sequence 95% or more identical to a second polynucleotide sequence selected from the group consisting of:
(a) a polynucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues m to 233 of SEQ ID NO:2, where m is an integer in the range of -26 to 27;

(b) a polynucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues -26 to x of SEQ ID NO:2, where x is an integer in the range of 123 to 233; and
(c) a polynucleotide sequence encoding a polypeptide having the amino acid sequence consisting of residues m to x of SEQ ID NO2, m and x are defined in (a) and (b) above;
wherein percentage identity is determined using the BESTFIT program with parameters that calculate identity over the full length of the second polynucleotide sequence and that allows gaps of up to 5% of the total number of nucleotides of said nucleotide sequence.

137. An isolated nucleic acid molecule comprising a first polynucleotide sequence 90% or more identical to a second polynucleotide sequence selected from the group consisting of:
(a) a polynucleotide sequence encoding a TRID (TRAIL receptor without intracellular domain) polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97798;
(b) a polynucleotide sequence encoding the mature TRID polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97798; and
(c) a polynucleotide sequence complementary to any of the polynucleotide sequences in (a) or (b) above; wherein percentage identity is determined using the BESTFIT program with parameters that calculate identity over the full length of the second polynucleotide sequence and that allows gaps of up to 10% of the total number of nucleotides of said nucleotide sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1, or to the nucleotide sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence

shown in SEQ ID NO:2, or to the amino acid sequence encoded by the deposited cDNA clone, can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2, or to the amino acid sequence encoded by the deposited cDNA clone, can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

United States Patent 6,284,486
Olsen , et al. September 4, 2001

This application hereby claims priority benefit to U.S. Appl. Ser. No. 60/033,869, filed Dec. 20, 1996 and U.S. Appl. Ser. No. 60/037,388, filed Feb. 7, 1997, which are hereby incorporated by reference.

62. An isolated polynucleotide comprising a first nucleic acid 95% or more identical to a reference nucleic acid encoding an amino acid sequence selected from the group consisting of:
(a) amino acids -20 to 142 of SEQ ID NO:2;
(b) amino acids -19 to 142 of SEQ ID NO:2;
(c) amino acids 1 to 142 of SEQ ID NO:2;
(d) the amino acid sequence of the mature polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97825; and
(e) the amino acid sequence of the complete polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97825;
wherein percent identity is calculated using Bestfit with the parameters set such that percentage of identity is calculated over the full length of the reference nucleic acid and that gaps in homology of up to 5% of the total number of nucleotides in the reference nucleic acid are allowed.

67. An isolated polynucleotide comprising a nucleic acid encoding an amino acid sequence 95% or more identical to a reference amino acid sequence selected from the group consisting of:
(a) amino acids -20 to 142 of SEQ ID NO:2;
(b) amino acids -19 to 142 of SEQ ID NO:2;
(c) amino acids 1 to 142 of SEQ ID NO:2;
(d) the amino acid sequence of the mature polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97825; and
(e) the amino acid sequence of the complete polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97825;
wherein percent identity is calculated using Bestfit with the parameters set such that percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference amino acid sequence are allowed.

68. An isolated polynucleotide comprising a nucleic acid which is 95% or more identical to a reference nucleic acid, wherein said reference nucleic acid is selected from the group consisting of:
(a) nucleotides 80 to 505 of SEQ ID NO:1;
(b) nucleotides 23 to 505 of SEQ ID NO:1; and
(c) nucleotides 20 to 505 of SEQ ID NO:1;

wherein percent identity is calculated using Bestfit with the parameters set such that percentage of identity is calculated over the full length of the reference nucleic acid and that gaps in homology of up to 5% of the total number of nucleotides in the reference nucleic acid are allowed.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 or to the nucleotide sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 or to the amino acid sequence encoded by the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

United States Patent 6,329,568
Gonsalves , et al. December 11, 2001

This application is a 371 of PCT/US94/01046 filed Jan. 27, 1994, which is a continuation of Ser. No. 08/010,410, filed Jan. 29, 1993, now abandoned.

23. An isolated DNA molecule comprising a nucleotide sequence having, over its entire length, above 80% similarity to the nucleotide sequence of SEQ. ID. No. 18, as measured using BESTFIT of the GCG sequence analysis software.

24. An isolated DNA molecule comprising a nucleotide sequence having, over its entire length, at least 80% similarity to the nucleotide sequence of SEQ. ID. No. 19, as measured using BESTFIT of the GCG sequence analysis software.

gene Com- Overall 53 K protein gene Intergenic 29 K protein
parisons.sup.a nt nt aa nt nt aa
B/ 76.4.sup.b 80.0 86.1 72.4 77.5 91.5(79.1)
CPNH1 (78.3).sup.c
B/L3 75.8 79.0 89.0(82.0) 76.4 78.0 91.1(79.9)
B/BL 76.3 -- -- 72.8 77.6 90.3(79.5)
B/I 63.0 -- -- -- 63.1 69.7(55.3)
CPNH1/L3 94.8 95.6 92.0(89.4) 89.2 96.8 99.6(98.5)
CPNH1/BL 96.4 -- -- 95.9 97.2 98.8(96.9)
CPNH1/I 62.7 -- -- -- 60.8 69.5(55.1)
L3/BL 95.1 -- -- 92.6 97.3 99.2(98.5)
L3/I 60.9 -- -- -- 60.9 69.5(55.1)
I/BL 61.7 -- -- -- 60.9 68.8(53.9)
.sup.a The partial or complete S RNA sequences of isolates TSWV-CPNH1 (2.916 kb), TSWV-L3 (2.837 kb), TSWV-BL (2.037 kb) and TSWV-I (1.144 kb) were used for comparisons with the S RNA sequence of the TSWV-B (3.049 kb).
.sup.b Percent similarities were calculated by Comparison of their nucleotide or predicted amino acid sequence using the program BESTFIT of the GCG Sequence analysis software package.
.sup.c Percent identity is in parenthesis.

United States Patent 6,388,052
Crabtree , et al. May 14, 2002

This application is a continuation application of Ser. No. 08/260,174 filed on Jun. 13, 1994, now U.S. Pat. No. 6,197,925 which is a continuation-in-part of U.S. Ser. No. 08/124,981 filed Sep. 20, 1993 (now U.S. Pat. No. 5,837,840).

1. An isolated polypeptide comprising an amino acid sequence which is at least 90% identical to at least 20 consecutive amino acids of SEQ ID NO: 38, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

2. The isolated polypeptide of claim 1, comprising an amino acid sequence which is at least 95% identical to at least 20 consecutive amino acids of SEQ ID NO: 38, wherein the percent identity is determined with the algorithm CAP, BESTFIT or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

15. The isolated polypeptide of claim 1, comprising an amino acid sequence which is at least 90% identical to at least 20 consecutive amino acids of the Rel Similarity Domain set forth in SEQ ID NO: 51, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

17. The polypeptide of claim 1, which comprises a Rel Similarity domain having an amino acid sequence which is at least about 73% identical to the amino acid sequence set forth in SEQ ID NO: 51, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

18. The polypeptide of claim 17, which comprises a Rel Similarity domain having an amino acid sequence which is at least about 90% identical to the amino acid sequence set forth in SEQ ID NO: 51, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by

computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

United States Patent 6,403,557
Greene , et al. June 11, 2002

This application is a continuation-in-part of, and claims the benefit of priority under 35 U.S.C. .sctn.120 to U.S. application Ser. No. 08/462,965, filed Jun. 5, 1995, now issued as U.S. Pat. No. 5,728,546 and PCT Application No. PCT/US095/07108, filed Jun. 5, 1995. This application also claims the benefit of priority under 35 U.S.C. .sctn.119(e) of U.S. Provisional Application Ser. Nos. 60/031,969 and 60/031,575, filed Nov. 27, 1996 and Dec. 4, 1996, respectively.

32. An isolated protein molecule comprising a first amino acid sequence having a least 95% identity to a second amino acid sequence selected from the group consisting of:
(a) amino acids +1 to +193 of SEQ ID NO:2;
(b) a mature portion of the protein encoded by the cDNA contained in ATCC Deposit No. 97148;
(c) amino acids -23 to +193 of SEQ ID NO:2; and
(d) the full length amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 97148,
wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said second amino acid sequence and that allow for gaps of homology of up to 5% of the total number of amino acid residues in said second amino acid sequence.

43. An isolated protein molecule comprising a first amino acid sequence having at least 95% identity to a second amino acid sequence selected from the group consisting of:
(a) amino acids residues n to +193 of SEQ ID NO:2, wherein n is an integer in the range of -23 to +10;
(b) amino acids residues -22 to m of SEQ ID NO:2, wherein m is an integer in the range of +154 to +192;
(c) amino acids n to m of SEQ ID NO:2, wherein n is an integer in the range of -23 to +10 and m is an integer in the range of +154 to +192; and
(d) a polypeptide consisting of a portion of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97148 wherein said portion excludes up to 33 amino acids from the amino terminus and up to 39 amino acids from the C-terminus of said complete amino acid sequence,
wherein % identity is determined using the Bestfit program with parameters that calculate % identity over the full length of said second amino acid sequence and that allow for gaps in homology of up to 5% of the total number of amino acids residues in said second amino acid sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in FIG. 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

By "% similarity" for two polypeptides is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711) and the default settings for determining similarity. Bestfit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2:482-489, 1981) to find the best segment of similarity between two sequences.

an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

Once a *prima facie* showing of no specific and substantial credible utility has been properly established, the applicant bears the burden of rebutting it. The applicant can do this by amending the claims, by providing reasoning or arguments, or by providing evidence in the form of a declaration under 37 CFR 1.132 or a patent or a printed publication that rebuts the basis or logic of the *prima facie* showing. If the applicant responds to the *prima facie* rejection, the Office personnel should review the original disclosure, any evidence relied upon in establishing the *prima facie* showing, any claim amendments, and any new reasoning or evidence provided by the applicant in support of an asserted specific and substantial credible utility. It is essential for Office personnel to recognize, fully consider and respond to each substantive element of any response to a rejection based on lack of utility. Only where the totality of the record continues to show that the asserted utility is not specific, substantial, and credible should a rejection based on lack of utility be maintained.

If the applicant satisfactorily rebuts a *prima facie* rejection based on lack of utility under § 101, withdraw the § 101 rejection and the corresponding rejection imposed under § 112, first paragraph.

Dated: December 29, 2000.

Q. Todd Dickinson,

Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office.

[FR Doc. 01-322 Filed 1-4-01; 8:45 am]

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DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

[Docket No. 991027288-0264-02]

RIN 0651-AB10

Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, "Written Description" Requirement

AGENCY: United States Patent and Trademark Office, Commerce.

ACTION: Notice.

SUMMARY: These Guidelines will be used by USPTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. These Guidelines supersede the "Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 'Written Description' Requirement" that were published in the **Federal Register** at 64 FR 71427, Dec. 21, 1999, and in the Official Gazette at 1231 O.G. 123, Feb. 29, 2000. These Guidelines reflect the current understanding of the USPTO regarding the written description requirement of 35 U.S.C. 112, ¶ 1, and are applicable to all technologies.

DATES: The Guidelines are effective as of January 5, 2001.

FOR FURTHER INFORMATION CONTACT: Stephen Walsh by telephone at (703) 305-9035, by facsimile at (703) 305-9373, by mail to his attention addressed to United States Patent and Trademark Office, Box 8, Washington, DC 20231, or by electronic mail at "stephen.walsh@uspto.gov"; or Linda Therkorn by telephone at (703) 305-8800, by facsimile at (703) 305-8825, by mail addressed to Box Comments, Commissioner for Patents, Washington, DC 20231, or by electronic mail at "linda.therkorn@uspto.gov."

SUPPLEMENTARY INFORMATION: As of the publication date of this notice, these Guidelines will be used by USPTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. Because these Guidelines only govern internal practices, they are exempt from notice and comment rulemaking under 5 U.S.C. 553(b)(A).

Discussion of Public Comments

Comments were received from 48 individuals and 18 organizations in response to the request for comments on the "Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 'Written Description' Requirement" published in the **Federal Register** at 64 FR 71427, Dec. 21, 1999, and in the Official Gazette at 1231 O.G. 123, Feb. 29, 2000. The written comments have been carefully considered.

Overview of Comments

The majority of comments favored issuance of final written description guidelines with minor revisions. Comments pertaining to the written description guidelines are addressed in detail below. A few comments addressed particular concerns with respect to the associated examiner training materials that are available for public inspection at the USPTO web site (www.uspto.gov). Such comments will be taken under advisement in the revision of the training materials; consequently, these comments are not specifically addressed below as they do not impact the content of the Guidelines. Several comments raised issues pertaining to the patentability of ESTs, genes, or genomic inventions with respect to subject matter eligibility (35 U.S.C. 101), novelty (35 U.S.C. 102), or obviousness (35 U.S.C. 103). As these comments do not pertain to the written description requirement under 35 U.S.C. 112, they have not been addressed. However, the aforementioned comments are fully addressed in the "Discussion of Public Comments" in the "Utility Examination Guidelines" Final Notice, which will be published at or about the same time as the present Guidelines.

Responses to Specific Comments

(1) *Comment:* One comment stated that the Guidelines instruct the patent examiner to determine the correspondence between what applicant has described as the essential identifying characteristic features of the invention and what applicant has claimed, and that such analysis will lead to error. According to the comment, the examiner may decide what applicant should have claimed and reject the claim for failure to claim what the examiner considers to be the invention. Another comment suggested that the Guidelines should clarify what is meant by "essential features of the invention." Another comment suggested that what applicant has identified as the "essential distinguishing characteristics" of the invention should be understood in terms of *Fiers* v. *Revel*, 984 F.2d 1164, 1169, 25 USPQ2d 1601, 1605 (Fed. Cir. 1993) ("Conception of a substance claimed *per se* without reference to a process requires conception of its structure, name,

formula, or definitive chemical or physical properties.").

Response: The suggestions have been adopted in part. The purpose of the written description analysis is to confirm that applicant had possession of what is claimed. The Guidelines have been modified to instruct the examiners to compare the scope of the invention claimed with the scope of what applicant has defined in the description of the invention. That is, the Guidelines instruct the examiner to look for consistency between a claim and what provides adequate factual support for the claim as judged by one of ordinary skill in the art from reading the corresponding written description.

(2) *Comment:* Two comments urge that *Regents of the University of California* v. *Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), is bad law and should not be followed by the USPTO because it conflicts with binding precedent, such as *Vas-Cath* v. *Mahurkar*, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991). *Response:* The final Guidelines are based on the Office's current understanding of the law and are believed to be fully consistent with binding precedent of the U.S. Supreme Court and the U.S. Court of Appeals for the Federal Circuit. *Eli Lilly* is a precedential decision by the Court that has exclusive jurisdiction over appeals involving patent law. Accordingly, the USPTO must follow *Eli Lilly*. Furthermore, the USPTO does not view *Eli Lilly* as conflicting with *Vas-Cath*. *Vas-Cath* explains that the purpose of the written description requirement is to ensure that the applicant has conveyed to those of skill in the art that he or she was in possession of the claimed invention at the time of filing. *Vas-Cath*, 935 F.2d at 1563–64, 19 USPQ2d at 1117. *Eli Lilly* explains that a chemical compound's name does not necessarily convey a written description of the named chemical compound, particularly when a genus of compounds is claimed. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1405. The name, if it does no more than distinguish the claimed genus from all others by function, does not satisfy the written description requirement because "it does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Thus, *Eli Lilly* identified a set of circumstances in which the words of the claim did not, without more, adequately convey to others that applicants had possession of what they claimed.

(3) *Comment:* Several comments urged that the Guidelines do not recognize the inconsistency between the original claim doctrine and the written description requirement as set out in *Fiers* and *Eli Lilly*. On the other hand, another comment asserts that there is no strong presumption that an originally filed claim constitutes an adequate written description of the claimed subject matter. Several comments indicate that *in haec verba* support should be sufficient to comply with the written description requirement. Two comments urge that the concept of constructive reduction to practice upon filing of an application has been ignored. *Response:* As noted above, the USPTO does not find *Fiers* and *Eli Lilly* to be in conflict with binding precedent. An original claim may provide written description for itself, but it still must be an *adequate* written description which establishes that the inventor was in possession of the invention. The "original claim doctrine" is founded on cases which stand for the proposition that originally filed claims are part of the written description of an application as filed, and thus subject matter which is present only in originally filed claims need not find independent support in the specification. *See, e.g., In re Koller*, 613 F.2d 819, 824, 204 USPQ 702, 706 (CCPA 1980) (later added claims of similar scope and wording were adequately described by original claims); *In re Gardner*, 480 F.2d 879, 880, 178 USPQ 149, 149 (CCPA 1973) ("Under these circumstances, we consider the original claim in itself adequate 'written description' of the claimed invention. It was equally a 'written description' * * * whether located among the original claims or in the descriptive part of the specification."). However, as noted in the preceding comment, *Eli Lilly* identified a set of circumstances in which the words of the claim did not, without more, adequately convey to others that applicants had possession of what they claimed. When the name of a novel chemical compound does not convey sufficient structural information about the compound to identify the compound, merely reciting the name is not enough to show that the inventor had possession of the compound at the time the name was written. The Guidelines indicate that there is a "strong presumption" that an adequate written description of the claimed invention is present when the application is filed, consistent with *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) ("we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims."). In most cases, the statement that "an originally filed claim is its own written description," is borne out because the claim language conveys to others of skill in the art that the applicant was "in possession" of what is claimed. The Guidelines emphasize that the burden of proof is on the examiner to establish that a description as filed is not adequate and require the examiner to introduce sufficient evidence or technical reasoning to shift the burden of going forward with contrary evidence to the applicant.

(4) *Comment:* One comment stated that the Guidelines change the substance of the written description requirement to require some level of enablement. The comment stated that the *Eli Lilly* case should not be followed because its change in the quality of the description required is in conflict with precedent. Another comment suggested that to comply with the written description requirement, the description must both (i) demonstrate possession of the claimed invention by the applicant; and (ii) put the public in possession of the claimed invention. *Response:* As noted in the comment above, the USPTO is bound by the Federal Circuit's decision in *Eli Lilly*. The Guidelines have been revised to clarify that an applicant must provide a description of the claimed invention which shows that applicant was in possession of the claimed invention. The suggestion to emphasize that the written description requirement must put the public in possession of the invention has not been adopted because it removes much of the distinction between the written description requirement and the enablement requirement. Although the two concepts are entwined, they are distinct and each is evaluated under separate legal criteria. The written description requirement, a question of fact, ensures that the inventor conveys to others that he or she had possession of the claimed invention; whereas, the enablement requirement, a question of law, ensures that the inventor conveys to others how to make and use the claimed invention.

(5) *Comment:* One comment suggested that the Guidelines should provide examples of situations in which the written description requirement was met but the enablement requirement was not, and vice versa. Another comment stated that examiners often use enablement language in making

written description rejections. *Response:* The enablement and written description requirements are not coextensive and, therefore, situations will arise in which one requirement is met but the other is not. Federal Circuit case law demonstrates many circumstances where enablement or written description issues, but not both, were before the Court. These Guidelines are intended to clarify for the examining corps the criteria needed to satisfy the written description requirement. For examples applying these Guidelines to hypothetical fact situations, see the "Synopsis of Application of Written Description Guidelines" (examiner training materials available on-line at *http://www.uspto.gov/web/menu/written.pdf).* These examples, as well as the examination form paragraphs and instructions on their proper use, provide the appropriate language examiners should use in making written description rejections.

(6) *Comment:* One comment disagreed with the statement in an endnote that "the fact that a great deal more than just a process is necessary to render a product invention obvious means that a great deal more than just a process is necessary to provide written description for a product invention." The comment indicated that the statement is overly broad and inconsistent with the "strong presumption that an adequate written description of the claimed invention is present when the application is filed." As an extreme case, for example, for product-by-process claims, nothing else would be needed to provide the written description of the product. *Response:* The endnote has been clarified and is now more narrowly drawn. However, there is no *per se* rule that disclosure of a process is sufficient to adequately describe the products produced by the process. In fact, *Fiers* v. *Revel* and *Eli Lilly* involved special circumstances where the disclosure of a process of making and the function of the product alone did not provide an adequate written description for product claims. Even when a product is claimed in a product-by-process format, the adequacy of the written description of the process to support product claims must be evaluated on a case-by-case basis.

(7) *Comment:* Several comments urge that actual reduction to practice, as a method of satisfying the written description requirement by demonstrating possession, has been over-emphasized. *Response:* The Guidelines have been clarified to state that describing an actual reduction to practice is one of a number of ways to show possession of the invention. Description of an actual reduction to practice offers an important "safe haven" that applies to all applications and is just one of several ways by which an applicant may demonstrate possession of the claimed invention. Actual reduction to practice may be crucial in the relatively rare instances where the level of knowledge and level of skill are such that those of skill in the art cannot describe a composition structurally, or specify a process of making a composition by naming components and combining steps, in such a way as to distinguish the composition with particularity from all others. Thus, the emphasis on actual reduction to practice is appropriate in those cases where the inventor cannot provide an adequate description of what the composition is, and a definition by function is insufficient to define a composition "because it is only an indication of what the [composition] does, rather than what it is." *Eli Lilly,* 119 F.3d at 1568, 43 USPQ at 1406. *See also Amgen Inc.* v. *Chugai Pharmaceutical Co.,* 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991).

(8) *Comment:* One comment asserts that the citation to *Pfaff* v. *Wells Electronics, Inc.,* 525 U.S. 55, 48 USPQ2d 1641 (1998) is inappropriate and should be deleted because *Pfaff* is concerned with § 102(b) on-sale bar, not written description. Another comment suggested that the Guidelines should provide an explanation of how the "ready for patenting" concept of *Pfaff* should be used in determining compliance with the written description requirement. *Response:* The Guidelines state the general principle that actual reduction to practice is not required to show possession of, or to adequately describe, a claimed invention (although, as noted in the previous comment, an actual reduction to practice is crucial in relatively rare instances). An alternative is to show that the invention described was "ready for patenting" as set out in *Pfaff.* For example, a *description* of activities that demonstrates the invention was "ready for patenting" satisfies the written description requirement. As *Wertheim* indicates, "how the specification accomplishes this is not material." 541 F.2d at 262, 191 USPQ at 96.

(9) *Comment:* One comment stated that the written description of a claimed DNA should be required to include the complete sequence of the DNA and claims should be limited to the DNA sequence disclosed. *Response:* Describing the complete chemical structure, *i.e.,* the DNA sequence, of a claimed DNA is one method of satisfying the written description requirement, but it is not the only method. *See Eli Lilly,* 119 F.3d at 1566, 43 USPQ2d at 1404 ("An adequate written description of a DNA * * * requires a precise definition, *such as* by structure, formula, chemical name, or physical properties." (emphasis added, internal quote omitted)). Therefore, there is no basis for a *per se* rule requiring disclosure of complete DNA sequences or limiting DNA claims to only the sequence disclosed.

(10) *Comment:* One comment stated that it is difficult to envision how one could provide a description of sufficient identifying characteristics of the invention without physical possession of a species of the invention, and thus this manner of showing possession should be considered as a way to show actual reduction to practice. *Response:* This suggestion has not been adopted. The three ways of demonstrating possession as set forth in the Guidelines are merely exemplary and are not mutually exclusive. While there are some cases where a description of sufficient relevant identifying characteristics will evidence an actual reduction to practice, there are other cases where it will not. *See, e.g., Ralston Purina Co.* v. *Far-Mar-Co, Inc.,* 772 F.2d 1570, 1576, 227 USPQ 177, 180 (Fed. Cir. 1985) (disclosure taken with the knowledge of those skilled in the art may be sufficient support for claims).

(11) *Comment:* One comment stated that the Guidelines should be revised to indicate that the test of disclosure of sufficiently detailed drawings should be expanded to include structural claiming of chemical entities. *Response:* The suggestion has been adopted.

(12) *Comment:* One comment stated that the Guidelines should reflect that an inventor is in possession of the invention when the inventor demonstrably has at least a complete conception thereof, and that factors and attributes which provide proof of written description should include evidence typically provided to prove a complete conception. *Response:* The suggestion has not been adopted because the conception analysis typically involves documentary evidence in addition to the description of the invention in the application as filed. However, it is acknowledged that if evidence typically provided to prove a complete conception is present in the specification as filed, it would be sufficient to show possession. The Federal Circuit has stated "[t]he conception analysis necessarily turns on the inventor's ability to describe his invention with particularity. Until he can do so, he cannot prove possession

of the complete mental picture of the invention." *Burroughs Wellcome Co.* v. *Barr Labs., Inc.*, 40 F.3d 1223, 1228, 32 USPQ2d 1915, 1919 (Fed. Cir. 1994). As further noted by the Federal Circuit, in order to prove conception, "a party must show possession of every feature recited in the count, and that every limitation of the count must have been known to the inventor at the time of the alleged conception." *Coleman* v. *Dines*, 754 F.2d 353, 359, 224 USPQ 857, 862 (Fed. Cir. 1985).

(13) *Comment:* One comment indicated that a "possession" test does not appear in Title 35 of the U.S. Code and is not clearly stated by the Federal Circuit. Therefore, it is recommended that patent examiners be directed to use existing judicial precedent to make rejections of claims unsupported by a statutory written description requirement. *Response:* While the Federal Circuit has not specifically laid out a "possession" test, the Court has clearly indicated that possession is a cornerstone of the written description inquiry. *See, e.g., Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991); *see also Purdue Pharma L.P.* v. *Faulding Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("[o]ne skilled in the art, reading the disclosure, must immediately discern the limitation at issue in the claims") (internal quote omitted). The possession test as set forth in the Guidelines is extrapolated from case law in a wide variety of technologies and is not intended to be limiting. Any rejections made by examiners will be made under 35 U.S.C. 112, ¶1, with supporting rationale. Final rejections are appealable if applicant disagrees and follows the required procedures to appeal.

(14) *Comment:* Two comments indicated that if the amino acid sequence for a polypeptide whose utility has been identified is described, then the question of possession of a class of nucleotides encoding that polypeptide can be addressed as a relatively routine matter using the understanding of the genetic code, and that the endnote addressing this issue should be revised. *Response:* The suggestion of these comments has been incorporated in the Guidelines and will be reflected in the training materials. However, based upon *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994), this does not mean that applicant was in possession of any particular species of the broad genus.

(15) *Comment:* One comment disagreed with an endnote which stated that a laundry list disclosure of moieties does not constitute a written description of every species in a genus. Specifically, the comment indicates that if the existence of a functional genus is adequately described in the specification, a laundry list of the species within that genus must satisfy the written description requirement. *Response:* The suggestion to revise the endnote will not be adopted. A lack of adequate written description problem arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosure. This was aptly demonstrated in *In re Bell* and *In re Baird* where possession of a large genus did not put a person of ordinary skill in the art in possession of any particular species. *See also Purdue Pharma*, 230 F.3d at 1328, 56 USPQ2d at 1487 (because the original specification did not disclose the later claimed concentration ratio was a part of the invention, the inventors cannot argue that they are merely narrowing a broad invention).

(16) *Comment:* One comment suggested that in the majority of cases, a single species will support a generic claim, and that the Guidelines should emphasize this point. *Response:* The suggestion has been adopted to a limited degree. The Guidelines now indicate that a single species may, in some instances, provide an adequate written description of a generic claim when the description of the species would evidence to one of ordinary skill in the art that the invention includes the genus. Note, however, *Tronzo* v. *Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998), where the species in the parent application was held not to provide written description support for the genus in the child application.

(17) *Comment:* One comment asserted that the Guidelines should focus on the compliance of the claims, not the specification, with the written description requirement. *Response:* This suggestion will not be adopted. "The specification shall contain a written description of the invention." 35 U.S.C. 112. The claims are part of the specification. *Id.*, ¶ 2. If an adequate description is provided, it will suffice "whether located among the original claims or in the descriptive part of the specification." *In re Gardner*, 480 F.2d 879, 880, 178 USPQ 149 (CCPA 1973). The entire disclosure, including the specification, drawings, and claims, must be considered.

(18) *Comment:* One comment asserted that the Guidelines confuse "new matter," 35 U.S.C. 132, with the written description requirement, and that the same standard for written description should be applied to both original claims and new or amended claims. *Response:* The Guidelines indicate that for both original and amended claims, the inquiry is whether one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention at the time the application was filed.

(19) *Comment:* One comment suggested that the second paragraph of the section pertaining to determining what the claim as a whole covers should be deleted because it relates more to compliance with § 112, second paragraph, than with the written description requirement. *Response:* This suggestion will not be adopted. The claims must be construed and all issues as to the scope and meaning of the claim must be explored during the inquiry into whether the written description requirement has been met. The concept of treating the claim as a whole is applicable to all criteria for patentability.

(20) *Comment:* One comment suggested a different order for the general analysis for determining compliance with the written description requirement, starting with reading the claim, then the specification, and then determining whether the disclosure demonstrates possession by the applicant. *Response:* This suggestion will not be adopted. The claims must be construed as broadly as reasonable in light of the specification and the knowledge in the art. *See In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). Then the disclosure must be evaluated to determine whether it adequately describes the claimed invention, i.e., whether it conveys to a person having ordinary skill in the art that the applicant had possession of what he or she now claims.

(21) *Comment:* Several comments suggested that the Guidelines are unclear with regard to how the examiner should treat the transitional phrase "consisting essentially of." The comments also suggested that the endnote that explains "consisting essentially of" does not make clear how the use of this intermediate transitional language affects the scope of the claim. Several comments stated that the USPTO does not have legal authority to treat claims reciting this language as open (equivalent to "comprising"). Another comment suggested that the phrase "clear indication in the specification" be replaced with "explicit or implicit indication." *Response:* The transitional phrase "consisting essentially of" "excludes

ingredients that would 'materially affect the basic and novel characteristics' of the claimed composition." *Atlas Powder Co.* v. *E.I. DuPont de Nemours & Co.*, 750 F.2d 1569, 1574, 224 USPQ 409, 412 (Fed. Cir. 1984). The basic and novel characteristics of the claimed invention are limited by the balance of the claim. *In re Janakirama-Rao*, 317 F.2d 951, 954, 137 USPQ 893, 896 (CCPA 1963). However, during prosecution claims must be read broadly, consistent with the specification. *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). Thus, *for purposes of searching for and applying prior art in a rejection under 35 U.S.C. 102 or 103*, if the specification or the claims do not define the "basic and novel" properties of the claimed subject matter (or if such properties are in dispute), the broadest reasonable interpretation consistent with the specification is that the basic and novel characteristics are merely the presence of the recited limitations. *See, e.g., Janakirama-Rao*, 317 F.2d at 954, 137 USPQ at 895–96. This does not indicate that the intermediate transitional language is never given weight. Applicants may amend the claims to avoid the rejections or seek to establish that the specification provides definitions of terms in the claims that define the basic and novel characteristics of the claimed invention which distinguish the claimed invention from the prior art. When an applicant contends that additional steps or materials in the prior art are excluded by the recitation of 'consisting essentially of,' applicant has the burden of showing that the introduction of additional steps or components would materially change the characteristics of applicant's invention. *In re De Lajarte*, 337 F.2d 870, 143 USPQ 256 (CCPA 1964). The language used in the Guidelines is consistent with *PPG Industries Inc.* v. *Guardian Industries Corp.*, 156 F.3d 1351, 1355, 48 USPQ2d 1351, 1355 (Fed. Cir. 1998) ("PPG could have defined the scope of the phrase 'consisting essentially of' for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics.").

(22) *Comment:* One comment stated that the written description should "disclose the invention," including why the invention works and how it was developed. *Response:* This suggestion has not been adopted. An inventor does not need to know how or why the invention works in order to obtain a patent. *Newman* v. *Quigg*, 877 F.2d 1575, 1581, 11 USPQ2d 1340, 1345 (Fed. Cir. 1989). To satisfy the enablement requirement of 35 U.S.C. 112, ¶1, an application must disclose the claimed invention in sufficient detail to enable a person of ordinary skill in the art to make and use the claimed invention. To satisfy the written description requirement of 35 U.S.C. 112, ¶ 1, the description must show that the applicant was in possession of the claimed invention at the time of filing. There is no statutory basis to require disclosure of why an invention works or how it was developed. "Patentability shall not be negatived by the manner in which the invention was made." 35 U.S.C. 103(a).

(23) *Comment:* One comment recommended that the phrases "emerging and unpredictable technologies" and "unpredictable art" be replaced with the phrase—inventions characterized by factors which are not reasonably predictable in terms of the ordinary skill in the art—. *Response:* The suggestion is adopted in part and the recommended phrase has been added as an alternative.

(24) *Comment:* One comment recommended that the phrase "conventional in the art" be replaced with—part of the knowledge of one of ordinary skill in the art—. *Response:* The suggestion is adopted in part and the recommended phrase has been added as an alternative. The standard of "conventional in the art" is supported by case law holding that a patent specification "need not teach, and preferably omits, what is well known in the art." *See Spectra-Physics, Inc.* v. *Coherent, Inc.*, 827 F.2d 1524, 1534, 3 USPQ2d 1737, 1743 (Fed. Cir. 1987); *Hybritech Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). *See also Atmel Corp.* v. *Information Storage Devices, Inc.*, 198 F.3d 1374, 1382, 53 USPQ2d 1225, 1231 (Fed. Cir. 1999).

(25) *Comment:* One comment recommended that the Guidelines be amended to state that the appropriate skill level for determining possession of the claimed invention is that of a person of *ordinary* skill in the art. *Response:* The comment has not been adopted. The statutory language itself indicates that compliance with the requirements of 35 U.S.C. 112, ¶1, is judged from the standard of "any person skilled in the art." It is noted, however, that the phrases "one of skill in the art" and "one of ordinary skill in the art" appear to be synonymous. *See, e.g., Union Oil Co.* v. *Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000) ("The written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow *persons of ordinary skill in the art* to recognize that [he or she] invented what is claimed.' Thus, § 112, ¶ 1, ensures that, as of the filing date, the inventor conveyed with reasonable clarity to *those of skill in the art* that he was in possession of the subject matter of the claims." (citations omitted, emphasis added)).

(26) *Comment:* One comment stated that an endnote misstates the relevant law in stating that, to show inherent written descriptive support for a claim limitation, the inherent disclosure must be such as would be recognized by a person of ordinary skill in the art. The comment recommended that the endnote be amended to delete the reference to recognition by persons of ordinary skill and to cite *Pingree* v. *Hull*, 518 F.2d 624, 186 USPQ 248 (CCPA 1975), rather than *In re Robertson*, 169 F.3d 743, 49 USPQ2d 1949 (Fed. Cir. 1999). *Response:* The comment has not been adopted. Federal Circuit precedent makes clear that an inherent disclosure must be recognized by those of ordinary skill in the art. *See, e.g., Hyatt* v. *Boone*, 146 F.3d 1348, 1354–55, 47 USPQ2d 1128, 1132 (Fed. Cir. 1998) ("[T]he purpose of the description requirement is 'to ensure that the inventor had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by him.' * * * Thus, the written description must include all of the limitations of the interference count, or the applicant must show that any absent text is necessarily comprehended in the description provided *and would have been so understood* at the time the patent application was filed." (emphasis added)). *See also Reiffin* v. *Microsoft Corp.*, 214 F.3d 1342, 1346, 54 USPQ2d 1915, 1917 (Fed. Cir. 2000) (The "application considered as a whole must convey to one of ordinary skill in the art, either explicitly or inherently, that [the inventor] invented the subject matter claimed * * *. See * * * *Continental Can Co. USA* v. *Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) (descriptive matter may be inherently present in a specification if one skilled in the art would necessarily recognize such a disclosure)").

(27) *Comment:* Several comments pointed out an inconsistency in the **Federal Register** Notice re: the Revised Interim Written Description Guidelines. The inconsistency concerned the treatment of claims directed to an isolated DNA comprising SEQ ID NO:1 wherein SEQ ID NO:1 is an expressed sequence tag. The comments contrasted paragraphs 34 and 35 of the Response to

Public Comments with the statement in the text of the Guidelines that a genus must be supported by a representative number of species (as analyzed in Example 7 of the training materials). *Response:* The USPTO acknowledges that there was an inconsistency. The Office notes that a claim reciting a nucleic acid comprising SEQ ID NO:1 may be subject to a rejection for lack of an adequate written description where particular identifiable species within the scope of the claim lack an adequate written description. The training materials as amended exemplify an appropriate analysis.

(28) *Comment:* One comment stated that the USPTO should respond to the issue of whether the U.S. is meeting its TRIPs obligations. This comment noted that the USPTO did not address an earlier comment regarding the "Interim Guidelines for the Examination of Patent Applications under the 35 U.S.C. 112, ¶ 1, 'Written Description' Requirement," 63 FR 32,639, June 15, 1998, which questioned whether the written description requirement is truly different from the enablement requirement, and indicated that such a requirement may be contrary to the TRIPs provisions of the World Trade Organization (Article 27.1). Article 27.1 requires WTO Members to, *inter alia*, make patents available, with limited exceptions, for products and processes in all fields of technology so long as those products and processes are new, involve an inventive step, and are capable of industrial application. The comment further suggested a response. *Response:* TRIPs Article 27 does not address what must be included in a patent application to allow WTO Member officials to determine whether particular inventions meet the standards for patentability established in that Article. TRIPs Article 29, which is more relevant to this comment, states that Members "shall require" patent applicants to disclose their invention "in a manner sufficiently clear and complete for the invention to be carried out by a person skilled in the art." If the written description is not clear and complete, the applicant may not have been in possession of the invention. This may support both written description and enablement standards. In addition, Article 29 expressly authorizes Members to require patent applicants to disclose the best method the inventor knows at the time of filing an application for carrying out the invention.

(29) *Comment:* Two comments commended the USPTO for eliminating the Biotechnology Specific Examples in the Revised Interim Written Description Guidelines and providing separate training materials. One comment indicated a need to reconfirm the examples set forth in the Interim Written Description Guidelines published in 1998. *Response:* The current training materials reflect the manner in which the USPTO interprets the Written Description Guidelines.

(30) *Comment:* Several comments addressed specific concerns about the examiner training materials. *Response:* The comments received with respect to the training materials will be taken under advisement as the Office revises the training materials in view of the revisions to the Guidelines. The specific comments will not be addressed herein as they do not impact the language of the Guidelines.

Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, "Written Description" Requirement

These "Written Description Guidelines" are intended to assist Office personnel in the examination of patent applications for compliance with the written description requirement of 35 U.S.C. 112, ¶ 1. This revision is based on the Office's current understanding of the law and public comments received in response to the USPTO's previous request for public comments on its Revised Interim Written Description Guidelines and is believed to be fully consistent with binding precedent of the U.S. Supreme Court, as well as the U.S. Court of Appeals for the Federal Circuit and its predecessor courts.

This revision does not constitute substantive rulemaking and hence does not have the force and effect of law. It is designed to assist Office personnel in analyzing claimed subject matter for compliance with substantive law. Rejections will be based upon the substantive law, and it is these rejections which are appealable. Consequently, any perceived failure by Office personnel to follow these Guidelines is neither appealable nor petitionable.

These Guidelines are intended to form part of the normal examination process. Thus, where Office personnel establish a *prima facie* case of lack of written description for a claim, a thorough review of the prior art and examination on the merits for compliance with the other statutory requirements, including those of 35 U.S.C. 101, 102, 103, and 112, is to be conducted prior to completing an Office action which includes a rejection for lack of written description. Office personnel are to rely on this revision of the Guidelines in the event of any inconsistent treatment of issues involving the written description requirement between these Guidelines and any earlier guidance provided from the Office.

I. General Principles Governing Compliance With the "Written Description" Requirement for Applications

The first paragraph of 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention * * *." This requirement is separate and distinct from the enablement requirement.[1] The written description requirement has several policy objectives. "[T]he 'essential goal' of the description of the invention requirement is to clearly convey the information that an applicant has invented the subject matter which is claimed."[2] Another objective is to put the public in possession of what the applicant claims as the invention.[3] The written description requirement of the Patent Act promotes the progress of the useful arts by ensuring that patentees adequately describe their inventions in their patent specifications in exchange for the right to exclude others from practicing the invention for the duration of the patent's term.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention.[4] An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention.[5] Possession may be shown in a variety of ways including description of an actual reduction to practice,[6] or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete,[7] or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention.[8] A question as to whether a specification provides an adequate written description may arise in the context of an original claim which is not described sufficiently, a new or amended claim wherein a claim limitation has been added or removed, or a claim to entitlement of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c).[9] Compliance with the written description requirement is a question of

fact which must be resolved on a case-by-case basis.[10]

A. Original Claims

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed.[11] However, the issue of a lack of adequate written description may arise even for an original claim when an aspect of the claimed invention has not been described with sufficient particularity such that one skilled in the art would recognize that the applicant had possession of the claimed invention.[12] The claimed invention as a whole may not be adequately described if the claims require an essential or critical feature which is not adequately described in the specification and which is not conventional in the art or known to one of ordinary skill in the art.[13] This problem may arise where an invention is described solely in terms of a method of its making coupled with its function and there is no described or art-recognized correlation or relationship between the structure of the invention and its function.[14] A lack of adequate written description issue also arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process.[15]

B. New or Amended Claims

The proscription against the introduction of new matter in a patent application[16] serves to prevent an applicant from adding information that goes beyond the subject matter originally filed.[17] Thus, the written description requirement prevents an applicant from claiming subject matter that was not adequately described in the specification as filed. New or amended claims which introduce elements or limitations which are not supported by the as-filed disclosure violate the written description requirement.[18] While there is no *in haec verba* requirement, newly added claim limitations must be supported in the specification through express, implicit, or inherent disclosure. An amendment to correct an obvious error does not constitute new matter where one skilled in the art would not only recognize the existence of the error in the specification, but also recognize the appropriate correction.[19] Deposits made after the application filing date cannot be relied upon to support additions to or correction of information in the application as filed.[20]

Under certain circumstances, omission of a limitation can raise an issue regarding whether the inventor had possession of a broader, more generic invention.[21] A claim that omits an element which applicant describes as an essential or critical feature of the invention originally disclosed does not comply with the written description requirement.[22]

The fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.[23]

II. Methodology for Determining Adequacy of Written Description

A. Read and Analyze the Specification for Compliance With 35 U.S.C. 112, ¶1

Office personnel should adhere to the following procedures when reviewing patent applications for compliance with the written description requirement of 35 U.S.C. 112, ¶1. The examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed;[24] however, with respect to newly added or amended claims, applicant should show support in the original disclosure for the new or amended claims.[25] Consequently, rejection of an original claim for lack of written description should be rare. The inquiry into whether the description requirement is met is a question of fact that must be determined on a case-by-case basis.[26]

1. For Each Claim, Determine What the Claim as a Whole Covers

Claim construction is an essential part of the examination process. Each claim must be separately analyzed and given its broadest reasonable interpretation in light of and consistent with the written description.[27] The entire claim must be considered, including the preamble language[28] and the transitional phrase.[29] The claim as a whole, including all limitations found in the preamble,[30] the transitional phrase, and the body of the claim, must be sufficiently supported to satisfy the written description requirement.[31]

The examiner should evaluate each claim to determine if sufficient structures, acts, or functions are recited to make clear the scope and meaning of the claim, including the weight to be given the preamble.[32] The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, ¶1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

2. Review the Entire Application to Understand How Applicant Provides Support for the Claimed Invention Including Each Element and/or Step

Prior to determining whether the disclosure satisfies the written description requirement for the claimed subject matter, the examiner should review the claims and the entire specification, including the specific embodiments, figures, and sequence listings, to understand how applicant provides support for the various features of the claimed invention.[33] The analysis of whether the specification complies with the written description requirement calls for the examiner to compare the scope of the claim with the scope of the description to determine whether applicant has demonstrated possession of the claimed invention. Such a review is conducted from the standpoint of one of skill in the art at the time the application was filed[34] and should include a determination of the field of the invention and the level of skill and knowledge in the art. Generally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement. Information which is well known in the art need not be described in detail in the specification.[35]

3. Determine Whether There is Sufficient Written Description to Inform a Skilled Artisan That Applicant was in Possession of the Claimed Invention as a Whole at the Time the Application Was Filed

a. Original claims. Possession may be shown in many ways. For example, possession may be shown, *inter alia*, by describing an actual reduction to practice of the claimed invention. Possession may also be shown by a clear depiction of the invention in detailed drawings or in structural chemical formulas which permit a person skilled in the art to clearly recognize that applicant had possession of the claimed invention. An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention.[36]

A specification may describe an actual reduction to practice by showing

that the inventor constructed an embodiment or performed a process that met all the limitations of the claim and determined that the invention would work for its intended purpose.[37] Description of an actual reduction to practice of a biological material may be shown by specifically describing a deposit made in accordance with the requirements of 37 CFR 1.801 *et seq.*[38]

An applicant may show possession of an invention by disclosure of drawings[39] or structural chemical formulas[40] that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole. The description need only describe in detail that which is new or not conventional.[41] This is equally true whether the claimed invention is directed to a product or a process.

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics[42] which provide evidence that applicant was in possession of the claimed invention,[43] *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.[44] What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail.[45] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.[46]

(1) For each claim drawn to a single embodiment or species:[47]

(a) Determine whether the application describes an actual reduction to practice of the claimed invention.

(b) If the application does not describe an actual reduction to practice, determine whether the invention is complete as evidenced by a reduction to drawings or structural chemical formulas that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole.

(c) If the application does not describe an actual reduction to practice or reduction to drawings or structural chemical formula as discussed above, determine whether the invention has been set forth in terms of distinguishing identifying characteristics as evidenced by other descriptions of the invention that are sufficiently detailed to show that applicant was in possession of the claimed invention.

(i) Determine whether the application as filed describes the complete structure (or acts of a process) of the claimed invention as a whole. The complete structure of a species or embodiment typically satisfies the requirement that the description be set forth "in such full, clear, concise, and exact terms" to show possession of the claimed invention.[48] If a complete structure is disclosed, the written description requirement is satisfied for that species or embodiment, and a rejection under 35 U.S.C. 112, ¶ 1, for lack of written description must not be made.

(ii) If the application as filed does not disclose the complete structure (or acts of a process) of the claimed invention as a whole, determine whether the specification discloses other relevant identifying characteristics sufficient to describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize applicant was in possession of the claimed invention.[49]

Whether the specification shows that applicant was in possession of the claimed invention is not a single, simple determination, but rather is a factual determination reached by considering a number of factors. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient.[50] Patents and printed publications in the art should be relied upon to determine whether an art is mature and what the level of knowledge and skill is in the art. In most technologies which are mature, and wherein the knowledge and level of skill in the art is high, a written description question should not be raised for original claims even if the specification discloses only a method of making the invention and the function of the invention.[51] In contrast, for inventions in emerging and unpredictable technologies, or for inventions characterized by factors not reasonably predictable which are known to one of ordinary skill in the art, more evidence is required to show possession. For example, disclosure of only a method of making the invention and the function may not be sufficient to support a product claim other than a product-by-process claim.[52] Furthermore, disclosure of a partial structure without additional characterization of the product may not be sufficient to evidence possession of the claimed invention.[53]

Any claim to a species that does not meet the test described under at least one of (a), (b), or (c) must be rejected as lacking adequate written description under 35 U.S.C. 112, ¶ 1.

(2) For each claim drawn to a genus:

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice (see (1)(a), above), reduction to drawings (see (1)(b), above), or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus (see (1)(c), above).[54]

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. On the other hand, there may be situations where one species adequately supports a genus.[55] What constitutes a "representative number" is an inverse function of the skill and knowledge in the art. Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. For inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species *cannot* be achieved by disclosing only one species within the genus.[56] Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.[57] If a representative number of adequately described species are not disclosed for a genus, the claim to that genus must be rejected as lacking adequate written description under 35 U.S.C. 112, ¶ 1.

b. New claims, amended claims, or claims asserting entitlement to the benefit of an earlier priority date or filing date under 35 U.S.C. 119, 120, or

365(c). The examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the original disclosure a description of the invention defined by the claims.[58] However, when filing an amendment an applicant should show support in the original disclosure for new or amended claims.[59] To comply with the written description requirement of 35 U.S.C. 112, ¶ 1, or to be entitled to an earlier priority date or filing date under 35 U.S.C. 119, 120, or 365(c), each claim limitation must be expressly,[60] implicitly,[61] or inherently[62] supported in the originally filed disclosure.[63] Furthermore, each claim must include all elements which applicant has described as essential.[64]

If the originally filed disclosure does not provide support for each claim limitation, or if an element which applicant describes as essential or critical is not claimed, a new or amended claim must be rejected under 35 U.S.C. 112, ¶ 1, as lacking adequate written description, or in the case of a claim for priority under 35 U.S.C. 119, 120, or 365(c), the claim for priority must be denied.

III. Complete Patentability Determination Under All Statutory Requirements and Clearly Communicate Findings, Conclusions, and Their Bases

The above only describes how to determine whether the written description requirement of 35 U.S.C. 112, ¶ 1, is satisfied. Regardless of the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of title 35 of the U.S. Code.

Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

A. For Each Claim Lacking Written Description Support, Reject the Claim Under Section 112, ¶ 1, for Lack of Adequate Written Description

A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption.[65] The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims.[66] In rejecting a claim, the examiner must set forth express findings of fact regarding the above analysis which support the lack of written description conclusion. These findings should:

(1) Identify the claim limitation at issue; and

(2) Establish a *prima facie* case by providing reasons why a person skilled in the art at the time the application was filed would not have recognized that the inventor was in possession of the invention as claimed in view of the disclosure of the application as filed. A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description.

When appropriate, suggest amendments to the claims which can be supported by the application's written description, being mindful of the prohibition against the addition of new matter in the claims or description.[67]

B. Upon Reply by Applicant, Again Determine the Patentability of the Claimed Invention, Including Whether the Written Description Requirement Is Satisfied by Reperforming the Analysis Described Above in View of the Whole Record

Upon reply by applicant, before repeating any rejection under 35 U.S.C. 112, ¶ 1, for lack of written description, review the basis for the rejection in view of the record as a whole, including amendments, arguments, and any evidence submitted by applicant. If the whole record now demonstrates that the written description requirement is satisfied, do *not* repeat the rejection in the next Office action. If the record still does not demonstrate that the written description is adequate to support the claim(s), repeat the rejection under 35 U.S.C. 112, ¶ 1, fully respond to applicant's rebuttal arguments, and properly treat any further showings submitted by applicant in the reply. When a rejection is maintained, any affidavits relevant to the 112, ¶ 1, written description requirement,[68] must be thoroughly analyzed and discussed in the next Office action.

Dated: December 29, 2000.

Q. Todd Dickinson,

Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office.

Endnotes

[1] *See, e.g., Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1114 (Fed. Cir. 1991).

[2] *In re Barker*, 559 F.2d 588, 592 n.4, 194 USPQ 470, 473 n.4 (CCPA 1977).

[3] *See Regents of the University of California* v. *Eli Lilly*, 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997), *cert. denied*, 523 U.S. 1089 (1998).

[4] *See, e.g., Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116. Much of the written description case law addresses whether the specification as originally filed supports claims not originally in the application. The issue raised in the cases is most often phrased as whether the original application provides "adequate support" for the claims at issue or whether the material added to the specification incorporates "new matter" in violation of 35 U.S.C. 132. The "written description" question similarly arises in the interference context, where the issue is whether the specification of one party to the interference can support the newly added claims corresponding to the count at issue, *i.e.*, whether that party can "make the claim" corresponding to the interference count. *See, e.g., Martin* v. *Mayer*, 823 F.2d 500, 503, 3 USPQ2d 1333, 1335 (Fed. Cir. 1987).

In addition, early opinions suggest the Patent and Trademark Office was unwilling to find written descriptive support when the only description was found in the claims; however, this viewpoint was rejected. *See In re Koller*, 613 F.2d 819, 204 USPQ 702 (CCPA 1980) (original claims constitute their own description); *accord In re Gardner*, 475 F.2d 1389, 177 USPQ 396 (CCPA 1973); *accord In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976) (accord). It is now well accepted that a satisfactory description may be in the claims or any other portion of the originally filed specification. These early opinions did not address the quality or specificity of particularity that was required in the description, *i.e.*, how much description is enough.

[5] *Lockwood* v. *American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

[6] An application specification may show actual reduction to practice by describing testing of the claimed invention or, in the case of biological materials, by specifically describing a deposit made in accordance with 37 CFR 1.801 *et seq. See also Deposit of Biological Materials for Patent Purposes, Final Rule*, 54 FR 34,864 (August 22, 1989) ("The requirement for a specific identification is consistent with the description requirement of the first paragraph of 35 U.S.C. 112, and to provide an antecedent basis for the biological material which either has been or will be deposited before the patent is granted." *Id.* at 34,876. "The description must be sufficient to permit verification that the deposited biological material is in fact that disclosed. Once the

patent issues, the description must be sufficient to aid in the resolution of questions of infringement." *Id.* at 34,880.). Such a deposit is not a substitute for a written description of the claimed invention. The written description of the deposited material needs to be as complete as possible because the examination for patentability proceeds solely on the basis of the written description. *See, e.g., In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985). *See also* 54 FR at 34,880 ("As a general rule, the more information that is provided about a particular deposited biological material, the better the examiner will be able to compare the identity and characteristics of the deposited biological material with the prior art.").

[7] *Pfaff* v. *Wells Electronics, Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

[8] *See Amgen, Inc.* v. *Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it").

[9] A description requirement issue can arise for original claims (*see, e.g., Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398) as well as new or amended claims. Most typically, the issue will arise in the context of determining whether new or amended claims are supported by the description of the invention in the application as filed (*see, e.g., In re Wright*, 866 F.2d 422, 9 USPQ2d 1649 (Fed. Cir. 1989)), whether a claimed invention is entitled to the benefit of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c) (*see, e.g., Tronzo* v. *Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998); *Fiers* v. *Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993); *In re Ziegler*, 992 F.2d 1197, 1200, 26 USPQ2d 1600, 1603 (Fed. Cir. 1993)), or whether a specification provides support for a claim corresponding to a count in an interference (*see, e.g., Fields* v. *Conover*, 443 F.2d 1386, 170 USPQ 276 (CCPA 1971)).

[10] *Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 (Fed. Cir. 1991).

[11] *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) ("we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims").

[12] See endnote 4.

[13] For example, consider the claim "A gene comprising SEQ ID NO:1." A determination of what the claim as a whole covers may result in a conclusion that specific structures such as a promoter, a coding region, or other elements are included. Although all genes encompassed by this claim share the characteristic of comprising SEQ ID NO:1, there may be insufficient description of those specific structures (*e.g.*, promoters, enhancers, coding regions, and other regulatory elements) which are also included.

[14] A biomolecule sequence described only by a functional characteristic, without any known or disclosed correlation between that function and the structure of the sequence, normally is not a sufficient identifying characteristic for written description purposes, even when accompanied by a method of obtaining the claimed sequence. For example, even though a genetic code table would correlate a known amino acid sequence with a genus of coding nucleic acids, the same table cannot predict the native, naturally occurring nucleic acid sequence of a naturally occurring mRNA or its corresponding cDNA. *Cf. In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993), and *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995) (holding that a process could not render the product of that process obvious under 35 U.S.C. 103). The Federal Circuit has pointed out that under United States law, a description that does not render a claimed invention obvious cannot sufficiently describe the invention for the purposes of the written description requirement of 35 U.S.C. 112. *Eli Lilly*, 119 F.3d at 1567, 43 USPQ2d at 1405.

Compare Fonar Corp. v. *General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805 (Fed. Cir. 1997) ("As a general rule, where software constitutes part of a best mode of carrying out an invention, description of such a best mode is satisfied by a disclosure of the functions of the software. This is because, normally, writing code for such software is within the skill of the art, not requiring undue experimentation, once its functions have been disclosed. * * * Thus, flow charts or source code listings are not a requirement for adequately disclosing the functions of software.").

[15] *See, e.g., Fujikawa* v. *Wattanasin*, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably lead" those skilled in the art to any particular species); *In re Ruschig*, 379 F.2d 990, 995, 154 USPQ 118, 123 (CCPA 1967) ("*If* n-propylamine had been used in making the compound instead of n-butylamine, the compound of claim 13 would have resulted. Appellants submit to us, as they did to the board, an imaginary specific example patterned on specific example 6 by which the above butyl compound is made so that we can see what a simple change would have resulted in a specific supporting disclosure being present in the present specification. The trouble is that there is no such disclosure, easy though it is to imagine it.") (emphasis in original); *Purdue Pharma L.P.* v. *Faulding Inc.*, 230 F.3d 1320, 1328, 56 USPQ2d 1481, 1487 (Fed. Cir. 2000) ("the specification does not clearly disclose to the skilled artisan that the inventors * * * considered the [] ratio to be part of their invention * * *. There is therefore no force to Purdue's argument that the written description requirement was satisfied because the disclosure revealed a broad invention from which the [later-filed] claims carved out a patentable portion").

[16] 35 U.S.C. §§ 132 and 251. *See also In re Rasmussen*, 650 F.2d 1212, 1214, 211 USPQ 323, 326 (CCPA 1981). *See* Manual of Patent Examining Procedure (MPEP) §§ 2163.06–2163.07 (7th Ed., Rev. 1, Feb. 2000) for a more detailed discussion of the written description requirement and its relationship to new matter.

[17] The claims as filed in the original specification are part of the disclosure and, therefore, if an application as originally filed contains a claim disclosing material not found in the remainder of the specification, the applicant may amend the specification to include the claimed subject matter. *In re Benno*, 768 F.2d 1340, 226 USPQ 683 (Fed. Cir. 1985).

[18] *See, e.g., In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971) (subgenus range was not supported by generic disclosure and specific example within the subgenus range); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily described by a genus encompassing it and a species upon which it reads).

[19] *In re Oda*, 443 F.2d 1200, 170 USPQ 260 (CCPA 1971). With respect to the correction of sequencing errors in applications disclosing nucleic acid and/or amino acid sequences, it is well known that sequencing errors are a common problem in molecular biology. *See, e.g.*, Peter Richterich, *Estimation of Errors in 'Raw' DNA Sequences: A Validation Study*, 8 Genome Research 251–59 (1998). If an application as filed includes sequence information and references a deposit of the sequenced material made in accordance with the requirements of 37 CFR § 1.801 *et seq.*, amendment may be permissible.

[20] Corrections of minor errors in the sequence may be possible based on the argument that one of skill in the art would have resequenced the deposited material and would have immediately recognized the minor error. Deposits made after the filing date can only be relied upon to provide support for the correction of sequence information if applicant submits a statement in compliance with 37 CFR § 1.804 stating that the biological material which is deposited is a biological material specifically defined in the application as filed.

[21] *See, e.g., Gentry Gallery, Inc.* v. *Berkline Corp.*, 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998) (claims to a sectional sofa comprising, *inter alia*, a console and a control means were held invalid for failing to satisfy the written description requirement where the claims were broadened by removing the location of the control means.); *Johnson Worldwide Associates* v. *Zebco Corp.*, 175 F.3d 985, 993, 50 USPQ2d 1607, 1613 (Fed. Cir. 1999) (In *Gentry Gallery*, the "court's determination that the patent disclosure did not support a broad meaning for the disputed claim terms was premised on clear statements in the written description that described the location of a claim element—the 'control means'—as 'the only possible location' and that variations were 'outside the stated purpose of the invention.' *Gentry Gallery*, 134 F.3d at 1479, 45 USPQ2d at 1503. *Gentry Gallery*, then, considers the situation where the patent's disclosure makes crystal clear that a particular (*i.e.*, narrow) understanding of a claim term is an 'essential element of [the inventor's] invention.' "); *Tronzo* v. *Biomet*, 156 F.3d at 1158–59, 47 USPQ2d at 1833 (Fed. Cir. 1998) (claims to generic cup shape were not entitled to filing date of parent application which disclosed "conical cup" in view of the disclosure of the

parent application stating the advantages and importance of the conical shape.).

[22] *See Gentry Gallery*, 134 F.3d at 1480, 45 USPQ2d at 1503; *In re Sus*, 306 F.2d 494, 504, 134 USPQ 301, 309 (CCPA 1962) ("[O]ne skilled in this art would not be taught by the written description of the invention in the specification that any 'aryl or substituted aryl radical' would be suitable for the purposes of the invention but rather that only *certain aryl radicals* and certain specifically substituted aryl radicals [*i.e.*, aryl azides] would be suitable for such purposes.") (emphasis in original). A claim which omits matter disclosed to be essential to the invention as described in the specification or in other statements of record may also be subject to rejection under 35 U.S.C. 112, ¶ 1, as not enabling, or under 35 U.S.C. 112, ¶ 2. *See In re Mayhew*, 527 F.2d 1229, 188 USPQ 356 (CCPA 1976); *In re Venezia*, 530 F.2d 956, 189 USPQ 149 (CCPA 1976); and *In re Collier*, 397 F.2d 1003, 158 USPQ 266 (CCPA 1968). *See also* MPEP § 2172.01.

[23] *See, e.g., Vas-Cath, Inc.*, 935 F.2d at 1563–64, 19 USPQ2d at 1117.

[24] *Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

[25] *See* MPEP §§ 714.02 and 2163.06 ("Applicant should * * * specifically point out the support for any amendments made to the disclosure."); and MPEP § 2163.04 ("If applicant amends the claims and points out where and/or how the originally filed disclosure supports the amendment(s), and the examiner finds that the disclosure does not reasonably convey that the inventor had possession of the subject matter of the amendment at the time of the filing of the application, the examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

[26] *See In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) ("Precisely how close [to the claimed invention] the description must come to comply with § 112 must be left to case-by-case development."); *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (inquiry is primarily factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure).

[27] *See, e.g., In re Morris*, 127 F.3d 1048, 1053–54, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997).

[28] "Preamble language" is that language in a claim appearing before the transitional phase, *e.g.*, before "comprising," "consisting essentially of," or "consisting of."

[29] The transitional term "comprising" (and other comparable terms, *e.g.*, "containing," "including," and "having") is "open-ended—it covers the expressly recited subject matter, alone or in combination with unrecited subject matter. *See, e.g., Genentech, Inc.* v. *Chiron Corp.*, 112 F.3d 495, 501, 42 USPQ2d 1608, 1613 (Fed. Cir. 1997) (" 'Comprising' is a term of art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim."); *Ex parte Davis*, 80 USPQ 448, 450 (Bd. App. 1948) ("comprising" leaves the "claim open for the inclusion of unspecified ingredients even in major amounts"). "By using the term 'consisting essentially of,' the drafter signals that the invention necessarily includes the listed ingredients and is open to unlisted ingredients that do not materially affect the basic and novel properties of the invention. A 'consisting essentially of' claim occupies a middle ground between closed claims that are written in a 'consisting of' format and fully open claims that are drafted in a 'comprising' format." *PPG Industries* v. *Guardian Industries*, 156 F.3d 1351, 1354, 48 USPQ2d 1351, 1353–54 (Fed. Cir. 1998). For the purposes of searching for and applying prior art under 35 U.S.C. 102 and 103, absent a clear indication in the specification or claims of what the basic and novel characteristics actually are, 'consisting essentially of' will be construed as equivalent to "comprising." *See, e.g., PPG*, 156 F.3d at 1355, 48 USPQ2d at 1355 ("PPG could have defined the scope of the phrase "consisting essentially of" for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics of the invention."). *See also In re Janakirama-Rao*, 317 F.2d 951, 954, 137 USPQ 893, 895–96 (CCPA 1963). If an applicant contends that additional steps or materials in the prior art are excluded by the recitation of "consisting essentially of," applicant has the burden of showing that the introduction of additional steps or components would materially change the characteristics of applicant's invention. *In re De Lajarte*, 337 F.2d 870, 143 USPQ 256 (CCPA 1964).

[30] *See Pac-Tec Inc.* v. *Amerace Corp.*, 903 F.2d 796, 801, 14 USPQ2d 1871, 1876 (Fed. Cir. 1990) (determining that preamble language that constitutes a structural limitation is actually part of the claimed invention).

[31] An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations. *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966.

[32] *See, e.g., Bell Communications Research, Inc.* v. *Vitalink Communications Corp.*, 55 F.3d 615, 620, 34 USPQ2d 1816, 1820 (Fed. Cir. 1995) ("[A] claim preamble has the import that the claim as a whole suggests for it."); *Corning Glass Works* v. *Sumitomo Elec. U.S.A., Inc.*, 868 F.2d 1251, 1257, 9 USPQ2d 1962, 1966 (Fed. Cir. 1989) (The determination of whether preamble recitations are structural limitations can be resolved only on review of the entirety of the application "to gain an understanding of what the inventors actually invented and intended to encompass by the claim.").

[33] An element may be critical where those of skill in the art would require it to determine that applicant was in possession of the invention. *Compare Rasmussen*, 650 F.2d at 1215, 211 USPQ at 327 ("one skilled in the art who read Rasmussen's specification would understand that it is unimportant *how* the layers are adhered, so long as they are adhered") (emphasis in original), *with Amgen, Inc.* v. *Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) ("it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it").

[34] *See, e.g., Wang Labs.* v. *Toshiba Corp.*, 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993).

[35] *See, e.g., Hybritech, Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379–80, 231 USPQ 81, 90 (Fed. Cir. 1986).

[36] *See, e.g., Purdue Pharma L.P.* v. *Faulding Inc.*, 230 F.3d 1320, ___, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) (the written description "inquiry is a factual one and must be assessed on a case-by-case basis"); *see also Pfaff* v. *Wells Electronics, Inc.*, 55 U.S. at 66, 119 S.Ct. at 311, 48 USPQ2d at 1646 ("The word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.' It is true that reduction to practice ordinarily provides the best evidence that an invention is complete. But just because reduction to practice is sufficient evidence of completion, it does not follow that proof of reduction to practice is necessary in every case. Indeed, both the facts of the *Telephone Cases* and the facts of this case demonstrate that one can prove that an invention is complete and ready for patenting before it has actually been reduced to practice.").

[37] *Cooper* v. *Goldfarb*, 154 F.3d 1321, 1327, 47 USPQ2d 1896, 1901 (Fed. Cir. 1998). *See also UMC Elecs. Co.* v. *United States*, 816 F.2d 647, 652, 2 USPQ2d 1465, 1468 (Fed. Cir. 1987) ("[T]here cannot be a reduction to practice of the invention * * * without a physical embodiment which includes all limitations of the claim."); *Estee Lauder Inc.* v. *L'Oreal, S.A.*, 129 F.3d 588, 593, 44 USPQ2d 1610, 1614 (Fed. Cir. 1997) ("[A] reduction to practice does not occur until the inventor has determined that the invention will work for its intended purpose."); *Mahurkar* v. *C.R. Bard, Inc.*, 79 F.3d 1572, 1578, 38 USPQ2d 1288, 1291 (Fed. Cir. 1996) (determining that the invention will work for its intended purpose may require testing depending on the character of the invention and the problem it solves).

[38] 37 CFR 1.804, 1.809. *See also* endnote 6.

[39] *See, e.g., Vas-Cath*, 935 F.2d at 1565, 19 USPQ2d at 1118 ("drawings alone may provide a 'written description' of an invention as required by § 112"); *In re Wolfensperger*, 302 F.2d 950, 133 USPQ 537 (CCPA 1962) (the drawings of applicant's specification provided sufficient written descriptive support for the claim limitation at issue); *Autogiro Co. of America* v. *United States*, 384 F.2d 391, 398, 155 USPQ 697, 703 (Ct. Cl. 1967) ("In those instances where a visual representation can flesh out words, drawings may be used in the same manner and with the same limitations as the specification.").

[40] *See, e.g., Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus.").

[41] *See Hybritech* v. *Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp.* v. *General Electric Co.*, 107 F.3d at 1549, 41 USPQ2d at 1805 (source code description not required).

[42] For example, the presence of a restriction enzyme map of a gene may be relevant to a statement that the gene has been isolated. One skilled in the art may be able to determine when the gene disclosed is the same as or different from a gene isolated by another by comparing the restriction enzyme map. In contrast, evidence that the gene could be digested with a nuclease would not normally represent a relevant characteristic since any gene would be digested with a nuclease. Similarly, isolation of an mRNA and its expression to produce the protein of interest is strong evidence of possession of an mRNA for the protein.

For some biomolecules, examples of identifying characteristics include a sequence, structure, binding affinity, binding specificity, molecular weight, and length. Although structural formulas provide a convenient method of demonstrating possession of specific molecules, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. For example, unique cleavage by particular enzymes, isoelectric points of fragments, detailed restriction enzyme maps, a comparison of enzymatic activities, or antibody cross-reactivity may be sufficient to show possession of the claimed invention to one of skill in the art. *See Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966 ("written description" requirement may be satisfied by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention").

[43] A definition by function alone "does not suffice" to sufficiently describe a coding sequence "because it is only an indication of what the gene does, rather than what it is." *Eli Lilly*, 119 F.3 at 1568, 43 USPQ2d at 1406. *See also Fiers*, 984 F.2d at 1169–71, 25 USPQ2d at 1605–06 (discussing *Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991)).

[44] If a claim limitation invokes 35 U.S.C. 112, ¶ 6, it must be interpreted to cover the corresponding structure, materials, or acts in the specification and "equivalents thereof." *See* 35 U.S.C. 112, ¶ 6. *See also B. Braun Medical, Inc.* v. *Abbott Lab.*, 124 F.3d 1419, 1424, 43 USPQ2d 1896, 1899 (Fed. Cir. 1997). In considering whether there is 35 U.S.C. 112, ¶ 1, support for a means- (or step) plus-function claim limitation, the examiner must consider not only the original disclosure contained in the summary and detailed description of the invention portions of the specification, but also the original claims, abstract, and drawings. A means- (or step-) plus-function claim limitation is adequately described under 35 U.S.C. 112, ¶ 1, if: (1) The written description adequately links or associates adequately described particular structure, material, or acts to the function recited in a means- (or step-) plus-function claim limitation; or (2) it is clear based on the facts of the application that one skilled in the art would have known what structure, material, or acts perform the function recited in a means- (or step-) plus-function limitation. Note also: A rejection under 35 U.S.C. 112, ¶ 2, "cannot stand where there is adequate description in the specification to satisfy 35 U.S.C. 112, first paragraph, regarding means-plus-function recitations that are not, per se, challenged for being unclear." *In re Noll*, 545 F.2d 141, 149, 191 USPQ 721, 727 (CCPA 1976). *See Supplemental Examination Guidelines for Determining the Applicability of 35 U.S.C. 112, ¶ 6*, 65 FR 38510, June 21, 2000.

[45] *See Hybritech Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94.

[46] *See, e.g., Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin* v. *Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [*i.e.*, "in the same words"] to be sufficient").

[47] A claim which is limited to a single disclosed embodiment or species is analyzed as a claim drawn to a single embodiment or species, whereas a claim which encompasses two or more embodiments or species within the scope of the claim is analyzed as a claim drawn to a genus. *See also* MPEP § 806.04(e).

[48] 35 U.S.C. 112, ¶ 1. *Cf. Fields* v. *Conover*, 443 F.2d 1386, 1392, 170 USPQ 276, 280 (CCPA 1971) (finding a lack of written description because the specification lacked the "full, clear, concise, and exact written description" which is necessary to support the claimed invention).

[49] For example, if the art has established a strong correlation between structure and function, one skilled in the art would be able to predict with a reasonable degree of confidence the structure of the claimed invention from a recitation of its function. Thus, the written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function. In contrast, without such a correlation, the capability to recognize or understand the structure from the mere recitation of function and minimal structure is highly unlikely. In this latter case, disclosure of function alone is little more than a wish for possession; it does not satisfy the written description requirement. *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 (written description requirement not satisfied by merely providing "a result that one might achieve if one made that invention"); *In re Wilder*, 736 F.2d 1516, 1521, 222 USPQ 369, 372–73 (Fed. Cir. 1984) (affirming a rejection for lack of written description because the specification does "little more than outline goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate"). *Compare Fonar*, 107 F.3d at 1549, 41 USPQ2d at 1805 (disclosure of software function adequate in that art).

[50] *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

[51] *See, e.g., In re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d 1527, 1534–35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992) ("One skilled in the art would know how to program a microprocessor to perform the necessary steps described in the specification. Thus, an inventor is not required to describe every detail of his invention. An applicant's disclosure obligation varies according to the art to which the invention pertains. Disclosing a microprocessor capable of performing certain functions is sufficient to satisfy the requirement of section 112, first paragraph, when one skilled in the relevant art would understand what is intended and know how to carry it out.")

[52] *See, e.g., Fiers* v. *Revel*, 984 F.2d at 1169, 25 USPQ2d at 1605; *Amgen.*, 927 F.2d at 1206, 18 USPQ2d at1021. Where the process has actually been used to produce the product, the written description requirement for a product-by-process claim is clearly satisfied; however, the requirement may not be satisfied where it is not clear that the acts set forth in the specification can be performed, or that the product is produced by that process.

[53] *See, e.g., Amgen*, 927 F.2d at1206, 18 USPQ2d at 1021 ("A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, *e.g.*, encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred, *i.e.*, until after the gene has been isolated.") (citations omitted). In such instances the alleged conception fails not merely because the field is unpredictable or because of the general uncertainty surrounding experimental sciences, but because the conception is incomplete due to factual uncertainty that undermines the specificity of the inventor's idea of the invention. *Burroughs Wellcome Co.* v. *Barr Laboratories Inc.*, 40 F.3d 1223, 1229, 32 USPQ2d 1915, 1920 (Fed. Cir. 1994). Reduction to practice in effect provides the only evidence to corroborate conception (and therefore possession) of the invention. *Id.*

[54] *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

[55] *See, e.g., Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326–27 (disclosure of a single method of adheringly applying one layer to another was sufficient to support a generic claim to "adheringly applying" because one skilled in the art reading the specification would understand that it is unimportant how the layers are adhered, so long as they are adhered); *In re Herschler*, 591 F.2d 693, 697, 200 USPQ 711, 714 (CCPA 1979) (disclosure of corticosteriod in DMSO sufficient to support claims drawn to a method of using a mixture of a "physiologically active steroid" and DMSO because "use of known chemical compounds in a manner auxiliary

to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds. Occasionally, a functional recitation of those known compounds in the specification may be sufficient as that description."); *In re Smythe*, 480 F.2d 1376, 1383, 178 USPQ 279, 285 (CCPA 1973) (the phrase "air or other gas which is inert to the liquid" was sufficient to support a claim to "inert fluid media" because the description of the properties and functions of the air or other gas segmentizing medium would suggest to a person skilled in the art that appellant's invention includes the use of "inert fluid" broadly.). However, in *Tronzo* v. *Biomet*, 156 F.3d at 1159, 47 USPQ2d at1833 (Fed. Cir. 1998), the disclosure of a species in the parent application did not suffice to provide written description support for the genus in the child application.

[56] *See, e.g., Eli Lilly.*

[57] For example, in the molecular biology arts, if an applicant disclosed an amino acid sequence, it would be unnecessary to provide an explicit disclosure of nucleic acid sequences that encoded the amino acid sequence. Since the genetic code is widely known, a disclosure of an amino acid sequence would provide sufficient information such that one would accept that an applicant was in possession of the full genus of nucleic acids encoding a given amino acid sequence, but not necessarily any particular species. *Cf. In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994).

[58] *See Wertheim*, 541 F.2d at 263, 191 USPQ at 97 ("[T]he PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

[59] *See* MPEP §§ 714.02 and 2163.06 ("Applicant should * * * specifically point out the support for any amendments made to the disclosure.").

[60] *See, e.g., In re Wright*, 866 F.2d 422, 425, 9 USPQ2d 1649, 1651 (Fed. Cir. 1989) (Original specification for method of forming images using photosensitive microcapsules which describes removal of microcapsules from surface and warns that capsules not be disturbed prior to formation of image, unequivocally teaches absence of permanently fixed microcapsules and supports amended language of claims requiring that microcapsules be "not permanently fixed" to underlying surface, and therefore meets description requirement of 35 U.S.C. 112.).

[61] *See, e.g., In re Robins*, 429 F.2d 452, 456–57, 166 USPQ 552, 555 (CCPA 1970) ("[W]here no explicit description of a generic invention is to be found in the specification * * * mention of representative compounds may provide an implicit description upon which to base generic claim language."); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily implicitly described by a genus encompassing it and a species upon which it reads).

[62] *See, e.g., In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950–51 (Fed. Cir. 1999) ("To establish inherency, the extrinsic evidence "must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'") (citations omitted).

[63] When an explicit limitation in a claim "is not present in the written description whose benefit is sought it must be shown that a person of ordinary skill would have understood, at the time the patent application was filed, that the description requires that limitation." *Hyatt* v. *Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998).

[64] *See, e.g., Johnson Worldwide Associates Inc.* v. *Zebco Corp.*, 175 F.3d at 993, 50 USPQ2d at 1613; *Gentry Gallery, Inc.* v. *Berkline Corp.*, 134 F.3d at 1479, 45 USPQ2d at 1503; *Tronzo* v. *Biomet*, 156 F.3d at 1159, 47 USPQ2d at 1833.

[65] *See, e.g., In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).

[66] *Wertheim*, 541 F.2d at 263, 191 USPQ at 97.

[67] *See Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326.

[68] *See In re Alton*, 76 F.3d 1168, 1176, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996).

[FR Doc. 01–323 Filed 1–4–01; 8:45 am]
BILLING CODE 3510–16–U

CORPORATION FOR NATIONAL AND COMMUNITY SERVICE

Revision of Currently Approved Information Collection; Comment Request

AGENCY: Corporation for National and Community Service

ACTION: Notice.

SUMMARY: The Corporation for National and Community Service (hereinafter "Corporation"), as part of its continuing effort to reduce paperwork and respondent burden, conducts a preclearance consultation program to provide the general public and Federal agencies with an opportunity to comment on proposed and/or continuing collections of information in accordance with the Paperwork Reduction Act of 1995 (PRA95) (44 U.S.C. 3506(c)(2)(A)). This program helps to ensure that requested data can be provided in the desired format, reporting burden (time and financial resources) is minimized, collection instruments are clearly understood, and the impact of collection requirement on respondents can be properly assessed.

Currently, the Corporation is soliciting comments concerning the proposed revision of its Voucher and Payment Request Form (OMB #3045–0014).

Copies of the forms can be obtained by contacting the office listed below in the address section of this notice.

DATES: Written comments must be submitted to the office listed in the **ADDRESSES** section by March 6, 2001.

ADDRESSES: Send comments to Levon Buller, National Service Trust, Corporation for National and Community Service, 1201 New York Ave., NW., Washington, DC 20525.

FOR FURTHER INFORMATION CONTACT: Levon Buller, (202) 606–5000, ext. 383.

SUPPLEMENTARY INFORMATION: The Corporation is particularly interested in comments which:

- Evaluate whether the proposed collection of information is necessary for the proper performance of the functions of the Corporation, including whether the information will have practical utility;
- Evaluate the accuracy of the agency's estimate of the burden of the proposed collection of information, including the validity of the methodology and assumptions used;
- Enhance the quality, utility and clarity of the information to be collected; and
- Minimize the burden of the collection of information on those who are to respond, including through the use of appropriate automated, electronic, mechanical, or other technological collection techniques or other forms of information technology, *e.g.*, permitting electronic submissions of responses.

Background

The Corporation supports programs that provide opportunities for individuals who want to become involved in national service. The service opportunities cover a wide range of activities over varying periods of time. Upon successfully completing an agreed-upon term of service in an approved AmeriCorps program, a national service participant—an AmeriCorps member—receives an "education award". This award is an amount of money set aside in the member's name in the National Service Trust Fund. This education award can be used to make payments towards qualified student loan or pay for educational expenses at qualified post-secondary institutions and approved school-to-work opportunities programs. Members have seven years in which to draw against any unused balance.

The National Service Trust is the office within the Corporation that administers the education award

to establish a claim of contributory or vicarious liability.

The district court stated that it was dismissing Softel's claim against Hodge pursuant to Fed. R. Civ. P. 50(a), under a directed verdict standard. However, Rule 50(a) relates to dismissals in jury trials, and this was a bench trial. At the time of this trial, in April 1991, dismissals in bench trials "on the ground that upon the facts and the law the plaintiff had not shown any right to relief" were handled under Rule 41(b) of the Federal Rules of Civil Procedure. 9 Charles Alan Wright & Arthur R. Miller, *Federal Practice and Procedure* § 2371 (1995). The substantive provisions of this rule were moved to Fed. R. Civ. P. 52(c) shortly afterwards, in December 1991. *See* 9A *id.* § 2573.1, at 493 n.1 (amendment effective Dec. 1, 1991).

Softel argues that we should take the trial court at its word and test its dismissal of this claim by the then-Rule 50(a) standard, i.e., whether, drawing every inference and making all credibility assessments in favor of Softel, Softel had presented sufficient evidence to support a verdict. Dragon argues that we should not review the trial court in this manner since the court in a bench trial has the power to make its own assessments of the evidence and probably did so here. We agree with Softel that the court made no findings but dismissed the case against Hodge by holding as a matter of law that Softel could not recover under any view of the evidence presented.

[8] However, this does not avail Softel because we also agree with the district court that, even drawing every inference in favor of Softel, Softel had at the close of its case failed to bring forward sufficient evidence to sustain its claims against Hodge. Therefore, no findings were necessary; indeed, they would have been an exercise in futility. To establish contributory infringement, Softel was required to show that Hodge "authorized the [infringing] use." *Sony Corp. v. Universal City Studios, Inc.*, 464 U.S. 417, 437 [220 USPQ 665] (1984). Softel points to no such showing prior to the district court's ruling, and offers no such evidence on appeal. To establish vicarious liability, Softel was required to show that Hodge had a "right and ability to supervise [that] coalesce[d] with an obvious and direct financial interest in the exploitation of copyrighted materials." *Shapiro, Bernstein & Co. v. H.L Green Co.*, 316 F.2d 304, 307 [137 USPQ 275] (2d Cir. 1963). The only evidence that Softel adduced prior to the court's ruling relating to the issue of Hodge's supervisory capacities and financial interests was that Hodge was the president of Dragon and a shareholder.[12] The evidence is too attenuated to establish a sufficiently "direct" financial interest in the exploitation of copyrighted materials. *Cf. id.* at 308 (finding appellee vicariously liable because, *inter alia*, it received 10%-12% of the sales of the infringing materials).

Therefore, the district court properly dismissed the claims against defendant Hodge.

III. CONCLUSION

The district court's dismissal of Softel's claims of non-literal infringement and trade secret misappropriation are vacated and remanded for further consideration consistent with this opinion. In all other respects, the district court's rulings are affirmed.

U.S. Court of Appeals
Federal Circuit

University of California v. Eli Lilly and Co.

No. 96-1175

Decided July 22, 1997

JUDICIAL PRACTICE AND PROCEDURE

1. Jurisdiction — Subject matter jurisdiction — In general (§405.0701)

Eleventh Amendment does not preclude trial, in Indiana, of patent infringement action brought by California state university in California federal district court, since Eleventh Amendment applies only to suits brought "against" state, not to suits brought "by" state, since instant case only involves university's patent claims and accused infringer's defenses, and does not involve any claim or counterclaim that places university

[12] Softel asserts that Dragon had only five employees, apparently in support of a theory that any president of a five-person corporation would have the requisite control and financial interest to establish vicarious liability. In support of this fact, Softel cites two documents in the Appendix on this appeal. First, it cites Judge Cannella's findings in *Softel II*, but these findings do not consider the number of employees at Dragon. Second, it cites a Dragon payroll report that does appear to indicate only five employees. Softel neglects to mention that this payroll report was entered into evidence more than four years after the ruling at issue here, in the damages phase of the trial, before a different judge. We therefore decline to consider it.

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NCLUSION

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RACTICE AND

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in position of defendant, and since case therefore does not raise Eleventh Amendment waiver issue, and consequently does not require determination of whether university waived its immunity only in California.

2. Jurisdiction — Venue; transfer of action — In patent actions (§405.1907)

Indiana federal district court did not abuse its discretion by affording too much weight to element of judicial economy in granting patent infringement defendant's motion to transfer case to Indiana, since fact that district court ultimately afforded little or no weight to other factors does not, standing alone, indicate abuse of discretion, and since, in case such as case at bar, in which several highly technical factual issues are presented and other relevant factors are in equipoise, interest of judicial economy may favor transfer to court that has become familiar with issues.

PATENTS

3. Patentability/Validity — Specification — Written description (§115.1103)

Patent specification does not provide adequate written description of claimed microorganism containing human insulin-encoding cDNA, since patent includes example providing process for obtaining human insulin-encoding cDNA, and describes protein that cDNA encodes, but provides no further information, such as sequence information indicating which nucleotides constitute human cDNA, pertaining to that cDNA's relevant structure or physical characteristics.

4. Patentability/Validity — Specification — Written description (§115.1103)

Patent's description of amino acid sequence of human insulin A and B chains fails to provide adequate written description of claimed microorganism containing human insulin-encoding cDNA, since description which renders claimed invention obvious is not sufficient to satisfy written description requirement of that invention, since claim to specific DNA is not made obvious by mere knowledge of desired protein sequence and methods for generating DNA that encodes that protein, and since description that does not render claimed invention obvious therefore does not sufficiently describe that invention for purposes of 35 USC 112.

5. Patentability/Validity — Specification — Written description (§115.1103)

In claims to genetic material, generic statement such as "vertebrate insulin cDNA" or "mammalian insulin cDNA," without more, is not adequate written description of claimed genus, since it does not distinguish genus from others except by function, and does not specifically define any of genes that fall within its definition, or describe structural features commonly possessed by members of genus that distinguish them from others; accordingly, naming type of material generally known to exist, in absence of knowledge as to what that material consists of, is not description of that material.

6. Patentability/Validity — Specification — Written description (§115.1103)

Patent does not provide adequate written description of claims generically reciting cDNA encoding vertebrate insulin and mammalian insulin, even though patent discloses rat insulin-encoding cDNA which is species within scope of generic claims, since cDNA is not defined or described by mere name "cDNA," even if accompanied by name of protein that it encodes, and though description of genus of cDNAs may be achieved by means of recitation of representative number of cDNAs, defined by nucleotide sequence, falling within scope of genus, or by recitation of structural features which are common to members of genus and constitute substantial portion of genus, claimed genera of vertebrate and mammal cDNA are not described by general language of patent's written description supported only by specific nucleotide sequence of rat insulin.

7. Practice and procedure in Patent and Trademark Office — Prosecution — Duty of candor — Materiality (§110.0903.04)

Infringement — Defenses — Fraud or unclean hands (§120.1111)

Patent for recombinant bacterial plasmids containing coding sequences of insulin genes is not unenforceable on ground that patent applicants allegedly misstated, in actual examples in application, that plasmid certified by National Institutes of Health had been used as cloning vector, when uncertified plasmid appears to have been used instead, since certified plasmid was not inoperable in stated examples, which therefore could have been stated as constructive rather than actual examples, since reasonable examiner would not have made any different decision if applicants had framed those examples as constructive examples, or if uncertified plasmid was recited in actual examples, and since misidentification of plasmid was therefore not material to patentability.

8. Infringement — Defenses — Prosecution history estoppel (§120.1105)

Claims for DNA transfer vector and microorganism containing human proinsulin genes are not infringed, either literally or under doctrine of equivalents, by defendant's production of recombinant fusion protein that is later cleaved to yield human PI, even though expression of human PI via fusion protein is described in patent, since record shows that plaintiff narrowed its claims, in response to prior art rejection, to exclude materials producing fusion protein, and since, in view of prosecution history, material used by defendant for expressing its fusion protein is not equivalent to asserted claims as matter of law.

9. Practice and procedure in Patent and Trademark Office — Prosecution — Duty of candor — Materiality (§110.0903.04)

European patent application was cumulative prior art to patent claiming DNA transfer vector and microorganism containing human proinsulin genes, and infringement plaintiff's failure to cite application to Patent and Trademark Office therefore did not render patent unenforceable for inequitable conduct, since examiner had already noted relevance of two articles describing essentially same work as that described in European application, since application's inclusion of claim directed in part to plasmid encoding human proinsulin did not add to its disclosure compared with cited references, and since European application was therefore not material to patentability, and cannot support inference of deceptive intent.

Particular patents — Chemical — Recombinant DNA

4,431,740, Bell, Pictet, Goodman, and Rutter, DNA transfer vector and transferred microorganisms containing human proinsulin and pre-proinsulin genes, judgment of unenforceability reversed; judgment of noninfringement affirmed.

4,625,525, Rutter, Pictet, Chirgwin, Goodman, Ullrich, and Shine, recombinant bacterial plasmids containing the coding sequences of insulin genes, judgment of unenforceability reversed; judgment of invalidity affirmed.

Appeal from the U.S. District Court for the Southern District of Indiana, Dillin, S.J.; 39 USPQ2d 1225.

Action by the University of California against Eli Lilly and Co. for patent infringement, one of six cases, consolidated for pretrial proceedings by Judicial Panel on Multidistrict Litigation under MDL docket no. 912, arising out of research arrangements and license agreements among University of California, Genentech Inc., and Eli Lilly and Co. From judgment holding both patents in suit unenforceable and not infringed, and holding one of those patents invalid as well, plaintiff appeals. Affirmed in part and reversed in part.

Harold J. McElhinny, of Morrison & Foerster, San Francisco, Calif.; Donald S. Chisum, Alan K. Palmer, Rachel Krevans, Gladys H. Monroy, and Debra A. Shetka, of Morrison & Foerster; Arthur I. Newstadt, Jean-Paul Lavalleye, Marc R. Labgold, and William J. Healey, of Oblon, Spivak, McClelland, Maier & Neustadt, Arlington, Va., for plaintiff-appellant.

Charles E. Lipsey, Donald R. Dunner, Howard W. Levine, and John R. Alison, of Finnegan, Henderson, Farabow, Garrett & Dunner, Washington, D.C.; Amy E. Hamilton, of Eli Lilly and Co., Indianapolis, Ind., for defendant-appellee.

Before Newman, Lourie, and Bryson, circuit judges.

Lourie, J.

The Regents of the University of California (UC) appeal from the judgment of the District Court for the Southern District of Indiana, holding that Eli Lilly & Company (Lilly) does not infringe U.S. Patent 4,652,525 or U.S. Patent 4,431,740 in its manufacture of human insulin; that the asserted claims of the '525 patent are invalid; and that both patents are unenforceable. *Regents of the Univ. of Cal. v. Eli Lilly and Co.*, 39 USPQ2d 1225 (S.D. Ind. 1995). We hold that the district court (1) properly exercised jurisdiction over this case for trial on the merits, (2) did not err in concluding that the asserted claims of the '525 patent are invalid for failure to provide an adequate written description of the subject matter of the asserted claims, and (3) did not clearly err in finding that Lilly did not infringe the '740 patent. We further hold that the district court (4) abused its discretion in holding that the '525 and '740 patents are unenforceable. We therefore affirm-in-part and reverse-in-part.

BACKGROUND

In 1990, UC brought this action in the Northern District of California, alleging that Lilly was infringing claims 1, 2, and 4-7 of the '525 patent under the doctrine of equivalents and infringing claims 2-3, 5-6, 8-10, and 13-14 of the '740 patent, either literally or under the doctrine of equivalents. Lilly responded that it does not infringe any of the asserted claims, that the asserted claims are invalid, and that the patents are unenforceable. Lilly did not assert any counterclaims against UC.

The patents in suit relate to recombinant DNA technology [1] and, more specifically, to recombinant plasmids and microorganisms that produce human insulin, a protein involved in the regulation of sugar metabolism. A person unable to produce insulin is afflicted with diabetes. Prior to the development of recombinant techniques for the production of human insulin, diabetic patients were treated with injections of animal insulin, which often caused allergic reactions. Human insulin produced by recombinant methods is less likely to produce such reactions. It consists of two separate amino acid chains, a 21-amino acid A chain and a 30-amino acid B chain, which are linked only by disulfide bonds. Healthy people produce insulin *in vivo* via the terminal enzymatic cleavage of preproinsulin (PPI) to yield proinsulin (PI), a single amino acid chain consisting of the A and B chains, linked by a sequence of additional amino acids that positions the A and B chains so that the disulfide bonds are readily formed. The PI is then further cleaved to liberate the linking sequence and yield insulin.

The '525 patent, the application for which was filed in May 1977, was based upon the determination of the PI and PPI cDNA sequences found in *rats*. Claim 1 of that patent reads as follows: "A recombinant *plasmid* replicable in procaryotic host containing within its nucleotide sequence a subsequence having the structure of the reverse transcript of an mRNA of a *vertebrate*, which mRNA encodes insulin." (emphasis added). Claim 2 relates to a recombinant procaryotic *microorganism* containing *vertebrate* insulin-encoding cDNA. Claims 4 and 5 depend from claim 2, and are limited, respectively, to *mammalian* and *human* insulin cDNA. Claim 6 depends from claim 1 and requires that the plasmid contain "at least one genetic determinant of the plasmid col E1." Claim 7 depends from claim 2 and requires that the microorganism be of a particular strain.

The '740 patent, the application for which was filed in September 1979, was based upon the determination of *human* PPI and PI cDNA sequences and the development of "tailoring" techniques for the incorporation of human PI cDNA into a recombinant plasmid. Using these techniques, a specific semi-synthetic DNA may be incorporated into a suitable transfer vector. Using one such tailoring technique, the human PI cDNA and the plasmid into which it is incorporated may be modified so that they contain complimentary oligo-dC and oligo-dG ends, which facilitate the formation of the recombinant plasmid. Independent claim 2 of the '740 patent reads: "A DNA transfer vector *comprising* an inserted cDNA *consisting essentially of* a deoxynucleotide sequence coding for human proinsulin, the plus strand of said cDNA having a defined 5' end, said 5' end being the first deoxynucleotide of the sequence coding for said proinsulin." (emphasis added). Dependent claim 3 is directed, *inter alia*, to a recombinant microorganism containing the transfer vector of claim 2. Claim 5 reads: "A DNA transfer vector *comprising* a deoxynucleotide sequence coding for human proinsulin *consisting essentially of* a plus strand having the sequence: [nucleotides that encode human proinsulin, described in structural terms]." (emphasis added). Claim 6 depends from claim 5 in the same manner that claim 3 depends from claim 2: it is directed to a recombinant microorganism containing the transfer vector of claim 5. Claim 8 is directed to an example of a human PI-encoding recombinant plasmid described in the specification; and claims 9 and 10, to microorganisms containing that plasmid. Claims 13 and 14 are directed to a subset of the transfer vector genus of claim 5 and accordingly depend from claim 5.

Lilly makes human PI using a semi-synthetic DNA to yield a cleavable *fusion protein* [2] that consists of a bacterial protein, a "cleavable linkage" consisting of a single methionine residue, and human PI. After the

[1] For a detailed discussion of recombinant DNA technology, see *Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1207-08 n.4, 18 USPQ2d 1016, 1022 n.4 (Fed. Cir. 1991) and *In re O'Farrell*, 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed. Cir. 1988) and references therein.

[2] For a detailed discussion of fusion proteins, see *Schendel v. Curtis*, 83 F.3d 1399, 1400 & n.3, 38 USPQ2d 1743, 1744 & n.3 (Fed. Cir. 1996).

fusion protein is produced, the desired human PI is obtained by cleaving it from the remainder of the fusion protein.

In 1992, pursuant to 28 U.S.C. § 1407 (1994), the Judicial Panel on Multidistrict Litigation (JPML) consolidated this case with five other related cases for pre-trial proceedings in the District Court for the Southern District of Indiana. *In re Recombinant DNA Tech. Patent and Contract Litig.*, No. 912 (J.P.M.L. Feb. 19, 1992). UC petitioned this court for a writ of mandamus, seeking to vacate the transfer order as barred by the Eleventh Amendment and inconsistent with various prior decisions in the consolidated cases, including two decisions of the District Court for the Northern District of California in this case. *See In re Regents of the Univ. of Cal.*, 964 F.2d 1128, 1131-32, 22 USPQ2d 1748, 1751-52 (Fed. Cir. 1992). We denied UC's petition, holding that the transfer did not force unconsented suit upon UC and thus was permissible for purposes of pretrial discovery. *Id.*, at 1134, 22 USPQ2d at 1754.

In 1994, responding to Lilly's pretrial motion, the District Court for the Southern District of Indiana transferred venue to itself for trial on the merits pursuant to 28 U.S.C. § 1404(a) (1994). After conducting a bench trial, the court issued a memorandum opinion in which it ruled, *inter alia*, that (1) Lilly does not infringe the asserted claims of either patent, 39 USPQ2d at 1228-39, (2) the asserted claims of the '525 patent, those directed to mammalian, vertebrate, and human cDNA, are invalid for lack of an adequate written description, *id.* at 1239-41, and (3) both patents are unenforceable due to inequitable conduct on the part of UC, *id.* at 1247-58. UC appeals from these rulings. We have jurisdiction pursuant to 28 U.S.C. § 1295(a)(1) (1994).

DISCUSSION

A. *Jurisdiction and Venue*

As a preliminary matter, UC argues that the District Court for the Southern District of Indiana lacked jurisdiction to hear this case on the merits and was an inappropriate venue for trial. UC first argues that the Eleventh Amendment deprives the Indiana court of jurisdiction. Specifically, UC asserts that by choosing to bring suit in the District Court for the Northern District of California, it waived its Eleventh Amendment immunity only in California federal courts. Relying on *Port Authority Trans-Hudson Corp. v. Feeney*, 495 U.S. 299, 307 (1990), UC argues that the Eleventh Amendment bars the transfer of this case for trial on the merits. Lilly responds that the Eleventh Amendment is inapplicable where, as here, a state asserts a claim and no counterclaim against the state is involved. We agree with Lilly that the Eleventh Amendment does not preclude trial in Indiana.

The Eleventh Amendment provides that: "The Judicial power of the United States shall not be construed to extend to any suit in law or equity, commenced or prosecuted against one of the United States by Citizens of another State, or by Citizens or Subjects of any Foreign State." U.S. Const. amend. XI. The Supreme Court has recently confirmed that "the reference to actions 'against one of the United States' encompasses not only actions in which a State is named as a defendant, but also certain actions against state agents and state instrumentalities," such as UC. *Regents of the Univ. of Cal. v. Doe*, 117 S. Ct. 900, 903 (1997); *see also BV Eng'g v. Univ. of Cal.*, 858 F.2d 1394, 1395, 8 USPQ2d 1421, 1422 (9th Cir. 1988).

The question raised by this case is whether it is one that has been brought "against" UC. In deciding this question, we are aided by the Supreme Court's guidance in its opinion in *United States v. Peters*, 9 U.S. (5 Cranch) 115 (1809) (Marshall, C.J.). In that case, the Court declined to apply the Eleventh Amendment to bar a suit instituted against the heirs of a deceased state treasurer. The Court stated:

> The right of a state to assert, as plaintiff, any interest it may have in a subject, which forms the matter in controversy between individuals, in one of the courts of the United States, is not affected by [the Eleventh] amendment; nor can [the amendment] be so construed as to oust the court of its jurisdiction, should such claim be suggested. The amendment simply provides, that no suit shall be commenced or prosecuted against a state. The state cannot be made a defendant to a suit brought by an individual; but it remains the duty of the courts of the United States to decide all cases brought before them by citizens of one state against citizens of a different state, where a state is not necessarily a defendant.

Id. at 139. This case involves a state's assertion of a claim rather than a state being a defendant.

[1] In the *Feeney* case relied on by UC, the Court applied the Eleventh Amendment because a claim for damages was asserted "against" a state instrumentality. The *Feeney* Court noted that "a State's Constitu-

tional immunity encompasses not merely *whether* it may be sued, but *where* it may be sued," 495 U.S. 299, 307 (quoting *Pennhurst State Sch. & Hosp. v. Halderman*, 465 U.S. 89, 99 (1984)), but the Court did not construe the Eleventh Amendment to apply to suits in which a state is solely a plaintiff, as UC is here. In fact, we do not believe that the Court has ever so construed the Eleventh Amendment. This is because the Eleventh Amendment applies to suits "against" a state, not suits by a state. Thus, we need not determine whether UC waived its immunity only in California, because this case does not create an Eleventh Amendment jurisdictional issue concerning which the question of waiver even arises. This case only involves UC's patent infringement claims and Lilly's defenses; it does not involve any claim or counterclaim *against* UC that places UC in the position of a defendant. Accordingly, we conclude that the Eleventh Amendment does not deprive the Indiana district court of jurisdiction in this case.

UC next argues that, under the law of the regional circuit to which appeal from the trial court would normally lie, the Indiana court abused its discretion by, as the court stated, transferring venue for trial on the merits from the California court to itself. *See Heller Fin., Inc. v. Midwhey Powder Co.*, 883 F.2d 1286, 1293 (7th Cir. 1987) (applying the abuse of discretion standard of review); *Lou v. Belzberg*, 834 F.2d 730, 739 (9th Cir. 1987) (same). Specifically, UC argues that the Indiana court abused its discretion by, *inter alia*, affording too much weight to the element of judicial economy in granting Lilly's motion to transfer the case to Indiana.[3] Lilly responds that the court acted within its discretion by retaining the case for trial and that it properly considered and weighed the relevant factors before deciding to do so.

[2] We agree with Lilly that the court did not err on this point. A federal district court may "[f]or the convenience of parties and witnesses, in the interest of justice, . . . transfer any civil action to any other district court or division where it might have been brought." 28 U.S.C. § 1404(a) (1994). The Indiana court based its decision to retain the case for trial on the merits on its finding that, although the convenience of the parties and witnesses did not favor either the Indiana or the California court, the interests of judicial economy would be served by trial in the Indiana court. Consideration of the interest of justice, which includes judicial economy, "may be determinative to a particular transfer motion, even if the convenience of the parties and witnesses might call for a different result." *Coffey v. Van Dorn Iron Works*, 796 F.2d 217, 220-21 (7th Cir. 1986); *Allen v. Scribner*, 812 F.2d 426, 436-37 (9th Cir. 1987) ("Because the transfer of this case undoubtedly would have led to delay, the district court did not abuse its discretion in denying Allen's motion notwithstanding possible inconvenience to the witnesses."); *Commodity Futures Trading Comm'n v. Savage*, 611 F.2d 270, 279 (9th Cir. 1979) (affirming denial of transfer motion because "[t]he district court was familiar with the case and transfer may have led to delay"). Thus, the fact that the district court ultimately afforded little or no weight to the other factors does not, standing alone, indicate that the district court abused its discretion. On the contrary, in a case such as this in which several highly technical factual issues are presented and the other relevant factors are in equipoise, the interest of judicial economy may favor transfer to a court that has become familiar with the issues. Accordingly, the court did not abuse its discretion by transferring the case after affording determinative weight to the consideration of judicial economy.

In its reply brief, UC first raises another basis for determining that Indiana was an improper venue for trial. UC argues that 28 U.S.C. § 1407(a) (1994) requires that a case transferred by the JPML for consolidated pretrial proceedings be returned for trial on the merits to the court from which it was transferred. Aware that it failed to address this issue in its opening brief in this appeal, UC contends that it adequately raised this argument when it filed its petition for mandamus seeking to vacate the transfer order for consolidation of discovery in Indiana. *See In re Regents*, 964 F.2d 1128, 22 USPQ2d 1748. Lilly first responds that UC waived this argument by failing to raise it in its opening brief in this appeal, regardless of the argument it made in its earlier petition. Lilly also maintains that the transfer was lawful, citing *In re American Continental Corp./Lincoln Savings & Loan Securities Litigation*, 102 F.3d 1524 (9th Cir. 1996), *cert. granted sub nom., Lexecon Inc. v. Milberg Weiss Bershad Hynes & Lerach*, 65 U.S.L.W. 3761 (U.S. May 20, 1997) (No. 96-1482), for the proposition that § 1407(a)

[3] UC also argues that the Indiana court abused its discretion by erroneously determining that UC could have brought this suit in Indiana without the state of California's consent, by overruling inconsistent decisions of the California district court, and by failing to give special weight to UC's choice of forum. We have considered these arguments and do not find them to be persuasive.

does not prohibit a discovery transferee court from transferring a case to itself for trial if an adequate reason for that transfer exists under 28 U.S.C. § 1404(a) (1994).

We agree with Lilly insofar as it argues that UC waived its argument regarding § 1407 by failing to raise it in its opening brief in this appeal. *See* Fed. R. App. P. 28(a)(6), 28(c); *Becton Dickinson & Co. v. C.R. Bard, Inc.*, 922 F.2d 792, 800, 17 USPQ2d 1097, 1103 (Fed. Cir. 1990) ("[A]n issue not raised by an appellant in its opening brief . . . is waived."). UC's assertion that it adequately raised this argument when it filed its petition for mandamus is not persuasive. In denying that petition, we noted that UC expressed concern that, *inter alia*, "Lilly will maneuver to try the merits of the California actions in Indiana . . . thus defeating [UC's] expectation and entitlement that the merits of the California actions will be tried in California." *In re Regents*, 964 F.2d at 1133, 22 USPQ2d at 1753. However, we declined to address UC's concern then because "[t]hese possibilities can not be evaluated in the abstract." *Id.* An assertion that the district court had actually erred was required, not the mere assertion that UC feared a potential error. We thus told UC that if it desired to contest the Indiana court's self-transfer, it would be required to raise that issue if and when the Indiana court actually transferred the case to itself. Because UC failed to do so by asserting error in a writ of mandamus or in its opening brief in this appeal, we decline to address the merits of its argument. Having determined that the Indiana court had jurisdiction and that its transfer of venue to itself under § 1404 was not, given the arguments properly before us, an abuse of that court's discretion, we address the remaining issues in UC's appeal.

B. *The '525 Patent*

1. *Validity*

The district court ruled that all of the claims of the '525 patent that UC asserted against Lilly, *viz.*, claims 1, 2, and 4-7, are invalid under § 112, ¶ 1, because the specification, although it provided an adequate written description of rat cDNA, did not provide an adequate written description of the cDNA required by the asserted claims. 39 USPQ2d at 1239-41.

Whether a specification complies with the written description requirement of § 112, ¶ 1, is a question of fact, which we review for clear error on appeal from a bench trial. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991); *Ralston Purina Co. v. Far-Mar-Co, Inc.*, 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985). To fulfill the written description requirement, a patent specification must describe an invention and do so in sufficient detail that one skilled in the art can clearly conclude that "the inventor invented the claimed invention." *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (1997); *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) ("[T]he description must clearly allow persons of ordinary skill in the art to recognize that [the inventor] invented what is claimed."). Thus, an applicant complies with the written description requirement "by describing the invention, with all its claimed limitations, not that which makes it obvious," and by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that set forth the claimed invention." *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966.

An adequate written description of a DNA, such as the cDNA of the recombinant plasmids and microorganisms of the '525 patent, "requires a precise definition, such as by structure, formula, chemical name, or physical properties," not a mere wish or plan for obtaining the claimed chemical invention. *Fiers v. Revel*, 984 F.2d 1164, 1171, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993). Accordingly, "an adequate written description of a DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a description of the DNA itself." *Id.* at 1170, 25 USPQ2d at 1606.

We first consider claim 5, which is specific to a microorganism containing a human insulin cDNA. UC argues that the district court clearly erred in finding that claim 5 is invalid under § 112, ¶ 1. Specifically, UC argues that a constructive or prophetic example in the '525 specification describes in sufficient detail how to prepare the claimed organism. Lilly responds that the district court properly applied the written description requirement, as this court applied it in *Fiers*, 984 F.2d at 1170-71, 25 USPQ2d at 1605-06, and thus did not clearly err in finding that the cDNA encoding human insulin required by claim 5 is not adequately described in the '525 patent.

[3] Claim 5 is directed to a recombinant procaryotic microorganism modified so that it contains "a nucleotide sequence having the structure of the reverse transcript of an mRNA of a [human], which mRNA encodes insulin." Thus, the definition of the claimed microorganism is one that requires

human insulin-encoding cDNA. The patent describes a method of obtaining this cDNA by means of a constructive example, Example 6. This example, however, provides only a general method for obtaining the human cDNA (it incorporates by reference the method used to obtain the rat cDNA) along with the amino acid sequences of human insulin A and B chains. Whether or not it provides an enabling disclosure, it does not provide a written description of the cDNA encoding human insulin, which is necessary to provide a written description of the subject matter of claim 5. The name cDNA is not itself a written description of that DNA; it conveys no distinguishing information concerning its identity. While the example provides a process for obtaining human insulin-encoding cDNA, there is no further information in the patent pertaining to that cDNA's relevant structural or physical characteristics; in other words, it thus does not describe human insulin cDNA. Describing a method of preparing a cDNA or even describing the protein that the cDNA encodes, as the example does, does not necessarily describe the cDNA itself. No sequence information indicating which nucleotides constitute human cDNA appears in the patent, as appears for rat cDNA in Example 5 of the patent. Accordingly, the specification does not provide a written description of the invention of claim 5.

[4] As indicated, Example 6 provides the amino acid sequence of the human insulin A and B chains, but that disclosure also fails to describe the cDNA. Recently, we held that a description which renders obvious a claimed invention is not sufficient to satisfy the written description requirement of that invention. *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966. We had previously held that a claim to a specific DNA is not made obvious by mere knowledge of a desired protein sequence and methods for generating the DNA that encodes that protein. *See, e.g., In re Deuel*, 51 F.3d 1552, 1558, 34 USPQ2d 1210, 1215 (1995) ("A prior art disclosure of the amino acid sequence of a protein does not necessarily render particular DNA molecules encoding the protein obvious because the redundancy of the genetic code permits one to hypothesize an enormous number of DNA sequences coding for the protein."); *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993). Thus, *a fortiori*, a description that does *not* render a claimed invention obvious does not sufficiently describe that invention for purposes of § 112, ¶ 1. Because the '525 specification provides only a general method of producing human insulin cDNA and a description of the human insulin A and B chain amino acid sequences that cDNA encodes, it does not provide a written description of human insulin cDNA. Accordingly, the district court did not err in concluding that claim 5 is invalid for failure to provide an adequate written description.

UC also argues that the district court erred in holding claims 1 and 2, which *generically* recite cDNA encoding vertebrate insulin, and claim 4, which is directed *generically* to cDNA encoding mammalian insulin, invalid. Dependent claims 6 and 7 similarly recite cDNA encoding vertebrate insulin. In support of this argument, UC cites the disclosure of a species (the rat insulin-encoding cDNA) within the scope of those generic claims. UC argues, citing *In re Angstadt*, 537 F.2d 498, 190 USPQ 214 (CCPA 1976) and *Utter v. Hiraga*, 845 F.2d 993, 6 USPQ2d 1709 (Fed. Cir. 1988), that because the '525 specification meets the requirements of § 112, ¶ 1, for a species within both of these genera, the specification necessarily also describes these genera. Lilly responds that the district court did not clearly err in finding that cDNA encoding mammalian and vertebrate insulin were not adequately described in the '525 patent, because description of one species of a genus is not necessarily a description of the genus.

We agree with Lilly that the claims are invalid. Contrary to UC's argument, a description of rat insulin cDNA is not a description of the broad classes of vertebrate or mammalian insulin cDNA. A written description of an invention involving a chemical genus, like a description of a chemical species, "requires a precise definition, such as by structure, formula, [or] chemical name," of the claimed subject matter sufficient to distinguish it from other materials. *Fiers*, 984 F.2d at 1171, 25 USPQ2d at 1606; *In re Smythe*, 480 F.2d 1376, 1383, 178 USPQ 279, 284-85 (CCPA 1973) ("In other cases, particularly but not necessarily, chemical cases, where there is unpredictability in performance of certain species or subcombinations other than those specifically enumerated, one skilled in the art may be found not to have been placed in possession of a genus. . . .").

The cases UC cites in support of its argument do not lead to the result it seeks. These cases do not compel the conclusion that a description of a species always constitutes a description of a genus of which it is a part. These cases only establish that every species in a genus need not be described in order that a genus meet the written description requirement. *See Utter*, 845 F.2d at 998-99, 6 USPQ2d at 1714 ("A specification may,

within the meaning of § 112 ¶ 1, contain a written description of a broadly claimed invention without describing all species that claim encompasses.") (affirming board's finding that an application that "describes in detail the geometry and components that make its *internal* pivot embodiment work" also sufficiently describes an interference count that is "silent as to the location of the pivot"). In addition, *Angstadt* is an enablement case and *Utter* involves machinery of limited scope bearing no relation to the complex biochemical claims before us.

[5] In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus. In claims to genetic material, however, a generic statement such as "vertebrate insulin cDNA" or "mammalian insulin cDNA," without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function. It does not specifically define any of the genes that fall within its definition. It does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus. A definition by function, as we have previously indicated, does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is. *See Fiers*, 984 F.2d at 1169-71, 25 USPQ2d at 1605-06 (discussing *Amgen*). It is only a definition of a useful result rather than a definition of what achieves that result. Many such genes may achieve that result. The description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention. *See In re Wilder*, 736 F.2d 1516, 1521, 222 USPQ 369, 372-73 (Fed. Cir. 1984) (affirming rejection because the specification does "little more than outlin[e] goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate."). Accordingly, naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of, is not a description of that material.

[6] Thus, as we have previously held, a cDNA is not defined or described by the mere name "cDNA," even if accompanied by the name of the protein that it encodes, but requires a kind of specificity usually achieved by means of the recitation of the sequence of nucleotides that make up the cDNA. *See Fiers*, 984 F.2d at 1171, 25 USPQ2d at 1606. A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus.[4] This is analogous to enablement of a genus under § 112, ¶ 1, by showing the enablement of a representative number of species within the genus. *See Angstadt*, 537 F.2d at 502-03, 190 USPQ at 218 (deciding that applicants "are *not* required to disclose *every* species encompassed by their claims even in an unpredictable art" and that the disclosure of forty working examples sufficiently described subject matter of claims directed to a generic process); *In re Robins*, 429 F.2d 452, 456-57, 166 USPQ 552, 555 (CCPA 1970) ("Mention of representative compounds encompassed by generic claim language clearly is not required by § 112 or any other provision of the statute. But, where no explicit description of a generic invention is to be found in the specification . . . mention of representative compounds may provide an implicit description upon which to base generic claim language."); *Cf. Gostelli*, 872 F.2d at 1012, 10 USPQ2d at 1618 (determining that the disclosure of two chemical compounds within a subgenus did not describe that subgenus); *In re Grimme*, 274 F.2d 949, 952, 124 USPQ 499, 501 (CCPA 1960) ("[I]t has been consistently held that the naming of one member of such a group is not, in itself, a proper basis for a claim to the entire group. However, it may not be necessary to enumerate a plurality of species if a genus is sufficiently identified in an application by 'other appropriate language.'") (citations omitted). We will not speculate in what other ways a broad genus of genetic material may be properly described, but it is clear to us, as it was to the district court, that the claimed genera of vertebrate and mammal cDNA are not described by the general language of the '525 patent's written description supported only by the specific nucleotide sequence of rat insulin.

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[4] We note that in claims 4, 5, and 12-14 of the '740 patent, genera of DNA sequences encoding human PI or PPI are described by reference to the structure of the claimed DNA sequences rather than by reference to their function.

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Accordingly, we reject UC's argument that the district court clearly erred in finding claims 1, 2, 4, 6, and 7 invalid for failure to provide an adequate written description. Because we affirm the district court's ruling that all of the claims of the '525 patent asserted against Lilly are invalid, we need not consider whether Lilly infringed those claims. *See B.F. Goodrich Co. v. Aircraft Braking Sys. Corp.*, 72 F.3d 1577, 1583, 37 USPQ2d 1314, 1319 (Fed. Cir. 1996).

2. *Enforceability*

The district court also ruled the '525 patent unenforceable on the ground of inequitable conduct. The court based this ruling on its findings that UC had violated National Institutes of Health (NIH) guidelines in order to develop the patented invention as soon as possible and had falsified material in its patent application in an effort to disguise its violation. The court noted that at the time the application that became the '525 patent was filed, NIH had certified only three plasmids for use with mammalian DNA: pSC101, pCR1, and pMB9. 39 USPQ2d at 1249. It then found that UC researchers knowingly used the uncertified pBR322 plasmid to hasten their determination of the rat PI and PPI cDNA sequences, and misrepresented that they had used pMB9, a certified plasmid, in the actual examples of their patent application. The court also found that a reasonable patent examiner would have viewed this misrepresentation as material to patentability. *Id.* at 1254.

UC argues that we should reverse the district court's ruling because it is based on a misinterpretation of the applicable law on inequitable conduct. Specifically, UC argues that the district court improperly considered alleged misrepresentations made to the NIH and Congress, and failed to properly consider whether the alleged misrepresentation in the patent application regarding the use of pMB9 was material to patentability. UC also argues that the district court clearly erred in finding that UC actually used pBR322 and then misrepresented that it used pMB9. In response, Lilly argues that under *General Electro Music Corp. v. Samick Music Corp.*, 19 F.3d 1405, 30 USPQ2d 1149 (Fed. Cir. 1994), UC's misrepresentation was sufficient to support a finding of inequitable conduct, and that such a misrepresentation need not bear directly on patentability as long as that misrepresentation was made in an effort to obtain a patent more quickly than otherwise. Lilly also argues that the district court properly found that UC's alleged pattern of deceit before a variety of governmental bodies was sufficient to render the patent unenforceable under the broad doctrine of "unclean hands." *See, e.g., Keystone Driller Co. v. General Excavator Co.*, 290 U.S. 240, 19 USPQ 228 (1933).

"A determination of inequitable conduct is committed to a district court's discretion. Accordingly, we review the district court's judgment for an abuse of discretion." *Kolmes v. World Fibers Corp.*, 107 F.3d 1534, 1541, 41 USPQ2d 1829, 1834 (Fed. Cir. 1997) (citing *Kingsdown Med. Consultants, Ltd. v. Hollister Inc.*, 863 F.2d 867, 876, 9 USPQ2d 1384, 1392 (Fed. Cir. 1988)). To overturn a discretionary ruling of a district court, "the appellant must establish that the ruling is based on clearly erroneous findings of fact or on a misapplication or misinterpretation of applicable law, or evidences a clear error of judgment on the part of the district court." *Molins PLC v. Textron, Inc.*, 48 F.3d 1172, 1178, 33 USPQ2d 1823, 1827 (Fed. Cir. 1995).

We conclude that the district court abused its discretion in holding the '525 patent to be unenforceable. An infringer asserting an inequitable conduct defense must demonstrate by clear and convincing evidence that the applicant or his attorney either failed to disclose material information or submitted false material information to the Patent and Trademark Office (PTO) and that the applicant or his attorney did so with an intent to deceive the PTO. *See Kingsdown*, 863 F.2d at 872, 9 USPQ2d at 1389. Information is material if a reasonable examiner would have considered it important to the patentability of a claim. *J.P. Stevens & Co. v. Lex Tex Ltd.*, 747 F.2d 1553, 1559, 223 USPQ 1089, 1092 (Fed. Cir. 1984).

[7] The alleged misinformation submitted to the PTO in this case consists of statements in Examples 4 and 5 of the specification that the pMB9 plasmid was used as the cloning vector for the rat cDNA when pBR322 appears to have been used. Lilly does not argue that the pMB9 plasmid was inoperable in the stated examples, only that Examples 4 and 5 should not have been stated as actual examples (even though they presumably could have been stated as constructive, *i.e.*, hypothetical, examples). Accordingly, Lilly must demonstrate that this distinction would have been considered material by a reasonable patent examiner. We conclude that it has not done so by clear and convincing evidence.

There is no reason to believe that a reasonable examiner would have made any different decision if UC had framed Examples 4 and 5 as constructive examples. *See Atlas Powder Co. v. E.I. Du Pont De Nemours &*

Co., 750 F.2d 1569, 1578, 224 USPQ 409, 415 (Fed. Cir. 1984) ("Even if intent could be inferred, and if the examples were constructive but not disclosed to the examiner as such, [the alleged infringer] has not shown the nondisclosure to have been material, *i.e.*, important to an examiner in allowing the patent to issue."); Manual of Patenting Examining Procedure (MPEP) § 707.07(l) (5th ed. 1993) ("The results of the tests and examples should not normally be questioned by the examiner unless there is a reasonable basis for questioning the results."); *cf. Consolidated Aluminum Corp. v. Foseco Int'l Ltd.*, 910 F.2d 804, 808-09, 15 USPQ2d 1481, 1484 (Fed. Cir. 1990) (affirming a finding of inequitable conduct based on an applicant's intentional disclosure of a "fictitious, inoperable" example and withholding of a best mode.). Moreover, the examiner would not have made any different decision if pBR322, the plasmid the district court found was actually used, was recited in the examples, because, as the record shows, the procedures described in Examples 4 and 5 for rat insulin cDNA worked to yield the intended results irrespective of whether pMB9 or pBR322 was used. The misidentification of the plasmid was therefore not material to patentability. Thus, no inequitable conduct occurred in the procurement of the patent.

In addition, contrary to the findings of the district court, a reasonable patent examiner would not have considered non-compliance with the NIH guidelines to be material to patentability. The district court based its finding of materiality on the theory that if the applicant had complied with the guidelines, the application might have been delayed and the applicants might not have been the first to apply for a patent on the claimed subject matter. However, such unfounded speculation is not clear and convincing evidence of materiality.

General Electro Music does not support Lilly's argument that UC's failure to have actually used pMB9 would have been material to patentability. In *General Electro Music*, we concluded that "a false statement in a petition to make special is material if, as here, it succeeds in prompting expedited consideration of the patent." 19 F.3d at 1411, 30 USPQ2d at 1154. We so concluded because, by filing a petition to make special, the applicant "requested special treatment and induced reliance on its statement that a prior art search had been conducted." *Id.* As explained above, UC's alleged mischaracterization of the pMB9 work as an actual example did not induce the examiner to act, or not to act, in reliance thereon. UC got no advantage in the patent examining process. Therefore, we conclude that the district court clearly erred in finding that the misidentification of the plasmid was material to patentability.

We also reject Lilly's alternative argument that the patent is unenforceable under the doctrine of "unclean hands." This court has previously refused to afford equitable relief in that guise in the absence of proof of materiality. In *J.P. Stevens*, 747 F.2d at 1560 n.7, 223 USPQ2d at 1093 n.7, we rejected the argument that "unclean hands" could render a patent unenforceable without proof of materiality because such a "categorization is inconsistent with this court's view that materiality is a necessary ingredient of any inequitable conduct." Accordingly, there is no legal basis for the conclusion that inequitable conduct occurred in the procurement of the patent and the district court therefore abused its discretion in its conclusion that the patent was unenforceable.

C. *The '740 Patent*

1. *Infringement*

The district court ruled that Lilly did not infringe claims 5-6 and 8-10 of the '740 patent either literally or under the doctrine of equivalents, 39 USPQ2d at 1231-38, and did not infringe claims 2-3 and 13-14 of the '740 patent under the doctrine of equivalents, *id.* at 1238. After evaluating the specification and the prosecution history, and receiving extrinsic evidence, the court construed these claims to be limited to genetic constructs (*i.e.*, "plasmids" and "transfer vectors") and microorganisms from which human PI is directly expressed. Accordingly, the court found that Lilly, which does not make or use such constructs or microorganisms, but expresses a recombinant fusion protein that is later cleaved to yield human PI, did not literally infringe the asserted claims. The court further determined that Lilly did not infringe the claims under the doctrine of equivalents because claim amendments made during the prosecution of the patent application bar UC from successfully asserting that the materials Lilly uses for expressing a recombinant fusion protein are equivalent to the claims of the '740 patent.

Challenging the district court's finding of a lack of literal infringement, UC argues that the district court incorrectly interpreted the claims. Specifically, UC argues that the use of the term "comprising" in the claims indicates that a transfer vector such as that used by Lilly will infringe the claims as long

as it includes the inserted cDNA encoding human PI, irrespective of the presence of other elements such as the DNA encoding the remainder of Lilly's fusion protein. Lilly responds that the district court correctly interpreted the claims in light of the prosecution history. Lilly argues that a prior art rejection was based on the examiner's conclusion that the prior art taught how to make recombinant insulin as part of a fusion protein and that UC therefore obtained allowance of the claims by specifically disclaiming transfer vectors that encode fusion proteins.

A determination of infringement requires a two-step analysis. "First, the claim must be properly construed to determine its scope and meaning. Second, the claim as properly construed must be compared to the accused device or process." *Carroll Touch, Inc. v. Electro Mechanical Sys., Inc.*, 15 F.3d 1573, 1576, 27 USPQ2d 1836, 1839 (Fed. Cir. 1993). The first step, claim construction, is a question of law which we review *de novo*; the proper construction of the claims is based upon the claim language, the specification, the prosecution history, and if necessary to aid the court's understanding of the patent, extrinsic evidence. *See Markman v. Westview Instruments, Inc.*, 52 F.3d 967, 979-81, 34 USPQ2d 1321, 1329-31 (Fed. Cir. 1995) (in banc), *aff'd*, 116 S. Ct. 1384, 38 USPQ2d 1461 (1996). The second step, determining whether a particular device infringes a properly construed claim, is a question of fact which we review for clear error on appeal from a bench trial. *See* Fed. R. Civ. P. 52(a); *Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1569, 219 USPQ 1137, 1140 (Fed. Cir. 1983). In order to prove infringement, a patentee must show that "the accused device includes every limitation of the [asserted] claim or an equivalent of each limitation." *Dolly, Inc. v. Spalding & Evenflo Cos.*, 16 F.3d 394, 397, 29 USPQ2d 1767, 1769 (Fed. Cir. 1994).

[8] We agree with Lilly that UC surrendered coverage of DNA that encodes a fusion protein. The district court correctly interpreted the asserted claims to be limited to genetic constructs and microorganisms that do not include DNA coding for a fusion protein. UC argues that the direct expression of human PI and the expression of human PI via a fusion protein are both described in the patent as part of the invention of the '740 patent, but that fact doesn't change the prosecution history which indicates that UC surrendered coverage of the latter in order to overcome prior art.

This surrender is best exemplified by the prosecution history relating to the claims that ultimately issued as claims 2 and 5. These claims as originally filed were directed, with varying degrees of specificity, to a DNA transfer vector *comprising* a DNA sequence coding for human PI. The word "comprising," as UC argues and as is well-established, permits inclusion of other moieties. However, during the prosecution of the patent, the examiner rejected these claims as unpatentable based on, *inter alia*, Ullrich *et al.*, 196 Science 1313 (June 17, 1977) and Villa-Komaroff *et al.*, 75 PNAS 3727 (August 1978).[5] The district court, essentially repeating the statements made by the patent examiner during the prosecution of the patent, found that these references taught,[6] respectively, the need "to combine the genetic information for the eukaryotic insulin gene with prokaryotic regulatory sequences, to obtain expression of insulin in bacteria," and "a general method for the expression and secretion of any eukaryotic protein [such as human PI] provided another protein . . . will serve as a carrier [as part of a fusion protein], by virtue of its leader sequence." 39 USPQ2d at 1232. The examiner thus rejected the claims because he believed that the prior art taught the use of recombinant eukaryotic/procaryotic fusion proteins for the production of a eukaryotic protein, including insulin, in a recombinant bacterium.

In an effort to overcome the rejection based on these references, UC first amended claim 2 to read, in pertinent part: "A DNA

[5] Several other publications of record before the PTO were found by the district court to teach the use of fusion proteins in the production of human PI. *See* 39 USPQ2d at 1231 n.12. For the sake of brevity, we do not discuss them here.

[6] UC also appears to argue that the district court clearly erred in finding that these references taught the production of human PI via a fusion protein. This argument misses the point of the analysis of prosecution history. As the Supreme Court recently noted, the question of the correctness of the examiner's rejection is "properly addressed on direct appeal from the denial of the patent, and will not be revisited in an infringement action." *Warner-Jenkinson Co. v. Hilton Davis Chem. Co.*, 117 S.Ct. 1040, 1051 n.7, 41 USPQ2d 1865, 1872-73 n.7 (1997). In construing the claims in view of prosecution history or in deciding whether to estop a patentee from asserting a certain range of equivalents, a court may only explore "the reason (right or wrong) for the objection and the manner in which the amendment addressed and avoided the objection." *Id.* Thus, the district court properly accepted the examiner's arguments for the purpose of construing the claims in view of the prosecution history.

transfer vector comprising an inserted cDNA *having* a [DNA] sequence coding for human [PI]. . . ." The word "having" still permitted inclusion of other moieties. When again confronted by a rejection based upon the same references and a later requirement that the word "having" be changed to "consisting essentially of," a narrower term, UC ultimately complied by amending claim 2 to its present form, *viz.*, "A DNA transfer vector comprising an inserted cDNA *consisting essentially of* a [DNA] sequence coding for human [PI]." Similarly, UC amended claim 5 to its present form, which reads, in pertinent part: "A DNA transfer vector comprising a [DNA] sequence coding for human [PI] *consisting essentially of* a plus strand having the sequence. . . ." (emphasis added). The examiner allowed these claims, noting that the required "consisting essentially of" language "excludes from the cDNA the presence of sequences other than [those coding for PI]." We agree with the district court that UC thus narrowed its claims in response to a prior art rejection to exclude the materials producing a fusion protein, as Lilly now does. UC urges us to read the examiner's statement on allowance of the claims narrowly as pertaining only to claim 2 and to exclude only DNA other than naturally-occurring human cDNA. However, that statement is not so limited; it expressly applies to claim 5 and, moreover, reflects the examiner's consistent requirement, acquiesced in by UC, that the DNA inserted in the claimed vectors code only for PI, not for a PI-containing fusion protein.[7]

We have considered all of the other arguments made by UC, including its assertion that the examiner's rejections were based on a distinction between tailored and non-tailored cDNA, but find them to be unpersuasive. In light of the prosecution history, we agree with the district court that claims 5 and 6, which contain the language added during prosecution, cannot be construed to literally cover Lilly's expression of human PI via a fusion protein. Furthermore, UC has stated in its appeal brief that, for purposes of the analysis of literal infringement, the scope of claims 8-10 is no broader than that of claims 5 and 6, and that it does not appeal the court's finding with respect to claims 8-10. Accordingly, we affirm the district court's construction of claims 5-6 and 8-10; its factual finding that Lilly does not literally infringe claims 5-6 is not clearly erroneous and is therefore also affirmed.

Regarding the district court's application of the doctrine of equivalents, UC argues that the district court improperly interpreted the prosecution history to indicate that UC had disclaimed vectors encoding fusion proteins instead of to indicate, as properly interpreted, that the claims were limited to "tailored" cDNA inserts. However, as indicated above, we find no error in the district court's interpretation of the claims and the prosecution history and hence its conclusion that Lilly does not infringe the asserted claims under the doctrine of equivalents.

When a claim has been narrowed by amendment for a "substantial reason related to patentability," such as to avoid a prior art rejection, the patentee may not assert that the surrendered subject matter is within the range of equivalents. *Warner-Jenkinson Co. v. Hilton Davis Chem. Co.*, 117 S.Ct. 1040, 1049-51, 41 USPQ2d 1865, 1871-73 (1997); *Insituform Techs., Inc. v. Cat Contracting, Inc.*, 99 F.3d 1098, 1107, 40 USPQ2d 1602, 1609 (Fed. Cir. 1996), *cert. denied*, 117 S. Ct. 1555 (1997); ("Prosecution history estoppel bars the patentee from recapturing subject matter that was surrendered by the patentee during prosecution in order to promote allowance of the claims."). "The application of prosecution history estoppel is a question of law subject to *de novo* review." *Id.*; *see also Warner-Jenkinson*, 117 S.Ct. at 1049-51, 41 USPQ2d at 1871-73.

As the district court properly concluded, the above-described prosecution history estops UC's '740 patent from dominating Lilly's expression of its fusion protein. As a matter of law, the material used by Lilly for expressing its fusion protein is not equivalent to that of the above-analyzed claims, or to the materials of the other asserted claims, *i.e.*, claims 2-3 and 13-14, for such an application of the doctrine of equivalents would allow UC to recapture subject matter it surrendered during the prosecution of the '740 patent. Accordingly, UC cannot meet its burden of establishing infringement under the doctrine of equivalents. The district court did not clearly err in determining that Lilly did not infringe the '740 patent, either literally or under the doctrine of equivalents.

2. *Enforceability*

The district court ruled that the '740 patent was unenforceable for inequitable con-

[7] UC's later-filed amendment pursuant to 37 C.F.R. § 1.312 (1983) ("Amendments after allowance"), in which it argued that the claims as allowed would not necessarily encompass the "trivial" oligo-dC and oligo-dG ends actually used to construct the plasmid of the '740 patent, also supports this broader reading of the examiner's statement.

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duct. 39 USPQ2d at 1255-58. The court based this ruling in part on its finding that UC failed to disclose to the PTO a highly--material reference, European Patent Application No. 1929 (EPA-1929), entitled "Plasmid for Transforming Bacterial Host to Render It Capable of Polypeptide Expression" in which the expression of human somatostatin and insulin are used as examples.[8] The court also based its ruling on its finding that UC was made aware of the materiality of EPA-1929 when it was cited as prior art by the European Patent Office (EPO) during the prosecution of the European counterpart of the application that led to the '740 patent. The court found that under these facts, it would "draw an inference of intent to mislead," *id.* at 1257, and accordingly, found that UC had engaged in inequitable conduct.

UC argues that it did not have a duty to disclose EPA-1929 to the PTO because it was merely cumulative of the references it had submitted to the PTO. Specifically, UC argues that EPA-1929 was cumulative of the two references on which EPA-1929 was based, which were already before the examiner when UC became aware of EPA-1929: Goeddel *et al.*, 76 PNAS 3727 (1979) and Itakura *et al.*, 198 Science 1056 (1977).[9] UC also argues that the district court misapplied the law on inequitable conduct by inferring an intent to deceive when the uncited reference was merely cumulative. Lilly responds that EPA-1929 was not cumulative because, unlike the reference before the examiner, it described a specific, enabling technique for making "tailored" DNA that would encode for a fusion protein including human PI. Lilly argues that UC's assertions of subjective good faith amount to no more than a mere denial of bad faith and accordingly that the district court properly disregarded those assertions. We agree with UC that the district court clearly erred in finding that EPA-1929 was not cumulative and, accordingly, in inferring an intent to deceive.

As stated above, we review a district court's ruling that a patent is unenforceable for inequitable conduct under an abuse of discretion standard. *Kingsdown Med. Consultants, Ltd. v. Hollister Inc.*, 863 F.2d 867, 876, 9 USPQ2d 1384, 1392 (Fed. Cir. 1988). An infringer asserting an inequitable conduct defense must prove by clear and convincing evidence that the applicant or his attorney failed to disclose material information or submitted false material information to the PTO, with an intent to deceive the PTO. *See id.* at 872, 9 USPQ2d at 1389. Information is material if a reasonable examiner would have considered it important to the patentability of a claim. *J.P. Stevens & Co. v. Lex Tex Ltd.*, 747 F.2d 1553, 1559, 223 USPQ 1089, 1092 (Fed. Cir. 1984). However, even where an applicant fails to disclose an otherwise material prior art reference, that failure will not support a finding of inequitable conduct if the reference is "simply cumulative to other references," *i.e.*, if the reference teaches no more than what a reasonable examiner would consider to be taught by the prior art already before the PTO. *Scripps Clinic & Research Found. v. Genentech, Inc.*, 927 F.2d 1565, 1582, 18 USPQ2d 1001, 1014 (Fed. Cir. 1991).

[9] The district court correctly found that UC knew of the materiality of EPA-1929 because the EPO considered EPA-1929 to be material to the examination of the European counterpart of the '740 patent. However, if EPA-1929 was merely cumulative of other references already before the examiner, UC's failure to cite it will not support a finding of inequitable conduct because one is justified in not submitting cumulative prior art. The record indicates that EPA-1929 was cumulative. The examiner had already noted the relevance of both the Itakura article, entitled "Expression in *Escherichia coli* of Chemically Synthesized Gene for the Hormone Somatostatin," and the Goeddel article, entitled "Expression in *Escherichia coli* of Chemically Synthesized Genes for Human Insulin." As is suggested by their respective titles and their dates of publication and submission, the work described in the two articles is essentially the same as that described in EPA-1929. In fact, the record indicates that the European patent examiner cited EPA-1929 against the European counterpart of the '740 patent, but cited the Goeddel article merely to demonstrate the state of the art and did not cite the Itakura article at all.

Lilly argues that these articles are distinguishable from EPA-1929 based on the fact that EPA-1929 also includes a claim (claim 6) directed, in part, to a plasmid encoding human proinsulin. But the inclusion of a claim is not controlling in a determination whether EPA-1929 is cumulative. What is relevant is whether EPA-1929 discloses subject matter relevant to the examination of

[8] This application was filed by Genentech, Inc. and named Drs. Itakura and Riggs as inventors.

[9] Drs. Itakura and Riggs, inventors of the EPA-1929 subject matter, are noted as authors on both of these articles.

the '740 patent application that is not taught by the Goeddel and Itakura articles. Plainly it does not. The Goeddel article and EPA-1929 *describe* in similar detail the same experiments which led to the production of a recombinant human insulin/β-galactosidase fusion protein. That Genentech attempted to claim a plasmid encoding human proinsulin in EPA-1929 does not add to its disclosure compared with the Goeddel article. We therefore conclude that the district court clearly erred in finding that EPA-1929 was not cumulative.

Because we conclude that the district court's finding of materiality was clearly erroneous, we also necessarily conclude that the district court clearly erred in inferring deceptive intent from the mere fact that UC did not cite EPA-1929. UC's failure to disclose the EPA-1929 reference, given its cumulative nature, is not clear and convincing evidence of inequitable conduct. Because the district court's conclusion that the '740 patent is unenforceable for inequitable conduct is based on clearly erroneous findings of materiality and intent, that conclusion is reversed.

CONCLUSION

The district court properly exercised jurisdiction over this case and did not abuse its discretion in transferring the case to itself for a trial on the merits. It did not clearly err in finding that the '525 patent does not provide an adequate written description of the subject matter of the asserted claims and thus properly held that those claims are invalid, nor did it clearly err in finding that Lilly did not infringe the asserted claims of the '740 patent. The court abused its discretion in holding that the '525 and '740 patents are unenforceable. Accordingly, the decision of the district court is

AFFIRMED-IN-PART and
REVERSED-IN-PART.

COSTS

Costs to Lilly.

U.S. Court of Appeals
First Circuit

The Saenger Organization Inc. v. Nationwide Insurance Licensing Associates Inc.

No. 96-2197

Decided July 18, 1997

COPYRIGHTS

1. Rights in copyright; infringement — Ownership of copyright — Works made for hire (§213.0305)

Record shows that infringement defendant prepared copyrighted insurance licensing texts both while he was plaintiff's employee, and within scope of his employment with plaintiff, since defendant's affidavits indicate that agreement he reached with plaintiff addressed issues of when and how long defendant would work on manuals, and that plaintiff retained right to control manner and means by which contested works were produced, since it is undisputed that defendant worked on manuals for plaintiff and at its specific behest, since record shows that plaintiff's office was important source of instrumentalities, tools, and human resources used in producing contested works, and indicates that defendant understood alleged oral agreement between parties to constitute employment agreement, and since six-year duration of defendant's work relationship with plaintiff, payment of salary to plaintiff during that period, provision of benefits, and defendant's tax treatment all favor finding employment relationship.

2. Rights in copyright; infringement — Ownership of copyright — Works made for hire (§213.0305)

Defendant's contention that alleged oral agreement between parties constituted partnership agreement regarding infringed insurance licensing texts is not supported by record evidence, since defendant's affidavits describe oral agreement only as one that delineated employment responsibilities and payment issues, and make no mention of intent by alleged principals to form partnership, since it is undisputed that defendant was not co-owner of plaintiff company, since defendant's allegation that agreement entitled him to equal share of net revenues from manuals, even if true, does not substantiate partnership theory, and since "partnership distributions" defendant alleges to have received pursuant to agreement were treated as compensation payments, rather than as partnership distribution income, for tax purposes.

3. Notice, deposit, and registration — Notice — Effect of publication; public display (§207.0303)

Notice, deposit, and registration — Registration — Effect (§207.0702)

Defendant's counterclaims for breach of agreement, fraud, and unfair business prac-

Evidentiary Appendix (g)

PRESS RELEASE #00-15
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PTO OFFERS TRAINING MATERIALS FOR INTERIM WRITTEN DESCRIPTION AND UTILITY GUIDELINES

The Patent and Trademark Office (PTO) today posted training materials designed to aid PTO's patent examiners in applying the interim written description and utility guidelines in a uniform and consistent manner to promote the issuance of high quality patents. The training materials will also assist patent applicants in responding to the PTO when utility or written description issues are raised during the examination of a patent application. The training materials can be found at http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf and http://www.uspto.gov/web/offices/pac/writtendesc.pdf. The guidelines were published in the Federal Register on December 21, 1999.

"I was very pleased with the breadth and the depth of the public comments that helped us shape our interim written description and utility guidelines," noted Q. Todd Dickinson, Commissioner of Patents and Trademarks. "I expect that the training materials we posted today will further inform the debate, supporting our efforts to strike the right balance with the final guidelines."

The Written Description training materials contain examples that are applicable to all areas of technology and all types of inventions. The examples include a variety of fact patterns and associated claims and conclude with a detailed claims analysis and recommended legal conclusions. Examples are provided in the mechanical, electrical, and biotechnological arts, and are based both on recent court decisions and typically encountered fact-patterns. The training materials emphasize that compliance with the written description requirement will be determined on a case-by-case basis. These materials serve to guide the examiners in determining whether the inventor has provided the necessary description of the invention.

The revised Interim Utility training materials focus on a three-pronged test for determining whether or not an invention is "useful" within the meaning of the law: Does the invention have a utility that is specific, substantial and credible? The materials include definitions of the elements that make up this test. A specific utility is one that is particular to the subject matter claimed. This contrasts with a general utility that would be applicable to the broad class of the invention.

A substantial utility is one that defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. A utility is credible unless the logic underlying the assertion is seriously flawed, or the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. All of the examples in the utility training materials are in the biotechnology or chemical arts.

The period for public comment on the revised Utility guidelines and the revised Interim Written Description guidelines continues until March 22, 2000, and all such comments are welcome.

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